This is to certify that the

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ALLELOPATHIC POTENTIAL OF ASPARAGUS

(Asparagus officinalis L.)

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# ALLELOPATHIC POTENTIAL OF ASPARAGUS (*Asparagus officinalis* L.)

By

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## ABSTRACT

## ALLELOPATHIC POTENTIAL OF ASPARAGUS (*Asparagus officinalis* L.)

By

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Two major problems of asparagus (*Asparagus officinalis* L.) are premature decline of fields and inability to replant asparagus after removal of old fields. Asparagus is believed to have allelopathic potential and compounds released from its tissues increase the incidence of *Fusarium* root rot on asparagus seedlings. Several plant species were inhibited differently by compounds released from dried root or fern tissues incubated in sterile or nonsterile soil under anaerobic or aerobic conditions. Both root and fern tissues were inhibitory to seedling development under anaerobic or aerobic conditions, as well as sterile or nonsterile soil conditions. Soil microorganisms were not necessary for, and over time, reduced asparagus tissue toxicity in soil. When excised asparagus roots were treated with increasing concentrations of water extracts from dried asparagus root tissues, electrolyte efflux was increased and respiration decreased. Exposure of asparagus seedlings to similar water extracts of asparagus root tissue decreased the peroxidase activity.

The responses of microbial populations in soils with dried asparagus root or fern tissue or planted with asparagus, *A. sprengeri*,

and other selected crops were compared. Fusarium spp. and other fungal populations increase 2 to 3 fold in treatments where asparagus root tissue was incorporated into soil. Although bacterial CFU's quickly increased in treatments where fern tissue was incorporated, both bacterial and fungal populations were reduced in soils where asparagus plants were grown compared to soils with other crops. The chloroform extracts of asparagus root tissues were separated using column, thin layer and high performance liquid chromatography. Ferulic, isoferulic, malic, citric, and fumaric acids were identified in HPLC fractions by GC-MS. NMR spectra of compounds isolated using preparative TLC or soxlet extractions also suggested that asparagusic acid and caffeic acid were present in root tissues.

## DEDICATION

This dissertation is dedicated to "The Abbott Bros. Band" of East Lansing, MI, including those greats, Bob M. Abbott, crack lead guitar, Dick W. Abbott, soulman, Barbara A. Abbott, Big mamma, and Brian H. Abbott, the jazz man - not forgetting great backup on bass, vocals, and guitar from their cousin, Michelle B. Abbott. I could never have made it without you guys. I tip my hat to you, Mr. Moore and Dr. Johnston.

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But most of all, I thank me because its done, by God, and I did it.

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# CHAPTER I

# LITERATURE REVIEW

## INTRODUCTION

### Asparagus Decline in Michigan

Asparagus (Asparagus officinalis L.) is an important vegetable crop grown on sandy soils that are not always considered suitable for other cash crops. Traditionally, it was assumed that if properly managed, plantations should stay commercially profitable for greater than 20 years (Tiedgens, 1924, 1926; Hanna, 1947; Lougheed and Tiessen, 1985). But, despite increased production due to increased acreage, yields of asparagus are currently declining, causing great consternation among asparagus growers. Today fields are being removed from production after 6 to 8 years due to sparse stands and small spear size resulting in low yields (Takatori and Souther, 1978). In 1978, the average yield for Michigan was 1,500 pounds per acre whereas in 1980, the state average was 900 pounds per acre (Anon, 1977, 1980). Today, only isolated commercial fields and experimental plots in Michigan report high yields.

Reduced yields from declining fields have also been reported for other production areas of the country. In New Jersey, asparagus production has dwindled from 30,000 acres to less than 1,000 acres (Herner and Vest, 1974). California, the largest producer of asparagus

in the United States, reports acreage planted to asparagus had decreased from 44,000 acres in 1974, to 28,000 acres in 1978 (Takatori and Souther, 1978). Declines in yields have also been reported for other asparagus growing areas of the world (Lougheed and Tiessen, 1985). In the Netherlands, acreage planted to asparagus decreased from 500 ha to 340 ha in 1970 (VanBakel and Kerstems, 1970).

Most asparagus fields are planted with approximately 10,000 crowns per acre, but 20% may be lost in the first five years after planting. In a 1978 field survey of asparagus fields in Michigan, the average crown population was 3,153 crowns per acre representing an overall 70% reduction in crown survival (Hodupp, 1983).

In addition to the decrease in longevity and productivity of etablished fields, experience has demonstrated that asparagus cannot be replanted in soils where decline forced removal of the crowns (Tiedgens, 1924, 1926; Hanna, 1947). Fields replanted immediately after plowing out crowns produced yields never more than one half the expected poundage, and when plants were direct seeded into old plantations, seedling mortality was practically 100% after 2-3 months (Hanna, 1947).

It is currently hypothesized that Fusarium crown and root rot is ultimately the causal factor in the decline in longevity and vigor, as well as inability to replant old asparagus fields (Cohen, 1946; Grogan and Kimble, 1959; Molot and Simone, 1965; Endo and Burkholder, 1971; Johnston, et. al. 1971). Decline is believed to be accelerated by environmental, biological, and physical stresses on the plants that may predispose them to infection by the Fusarium crown and/or root rot organisms (Farrish, 1939; Takatori, et. al. 1970, 1974; Hartung and Stephens, 1983, 1984; Evans and Stephens, 1984, 1985; Evans , et. al.,

1985; Robb, 1984). The Fusarium species implicated in the disease on asparagus are F. oxysporum (Schlecht.) emend. Snyder and Hansen f. sp. asparagi Cohen, and F. moniliforme (Sheldon) emend. Snyder and Hansen.

The Fusarium spp. are among the most cosmopolitan of the fungi and are of great economic importance since they play a major role in reducing yields and quality of many important food crops of the world (Nelson, et. al., 1981). The fusaria are capable of surviving in the soil almost indefinitely as chlamydospores or other resting structures (Nelson et. al., 1937; Booth, 1971; Nyvall and Kommedahl, 1968). They are facultative parasites which colonize living and non-living host tissue and are also capable of invading non-host tissues (Alexander, 1961; Hendrix and Nielson, 1958). Many of the modern fusarium diseases such as corn stalk rot are caused by a complex of organisms aided and abetted by a plethora of environmental factors and cultural practices (Toussoun and Patrick, 1963; Nelson, et. al.,1981). F. oxysporum is the most frequently isolated of the fusarium species in soils and is very active as a saphrophyte (Nelson, et. al. 1981). F. moniliforme is distributed throughout the world but is most common in the warmer regions. It is a major parasite on several species of the Poaceae, particularly rice. It is often found in association with other fungal organisms, particularly F. oxysporum, acting in consort with them to produce disease (Hendrix and Nielson, 1958; Booth, 1981; Nelson, et. al. 1981).

Fusarium crown and root rot is considered to be the most common disease of asparagus in the United States (Walker, 1952). The above-ground symptoms caused by both pathogens include yellowing, stunting and/or wilting of the ferns. The ferns may die in various stange of

elongation (Cohen, 1946, Endo and Burkeholder, 1971). Symptoms on asparagus associated with F. oxysporum f. sp. asparagi are elliptical reddish-brown lesions found at the base of the ferns and vascular discoloration within stems, roots and crowns. F. moniliforme causes dry crown rot and brown stem pith discoloration but no vascular discoloration (Johnston, et. al., 1979). Total root collapse can occur when seedlings are inoculated with F. moniliforme. F. moniliforme was once considered primarily important in older asparagus plantations, but recent research indicates this pathogen may also be the primary pathogen in one-year old crowns (Damicone and Manning, 1985).

Efforts to develop chemical controls for the Fusarium pathogens on asparagus have not proven successful. Soil fumigation and seed treatment reduce but do not eliminate decline symptoms (Damicone and Cooley, 1981; Manning and Vardaro, 1977; Lacy, 1979). Control is also confounded by the perennial nature of asparagus and the difficulty of combatting the pathogen in the soil without damaging the plant. In crops already studied, the most successful strategy for Fusarium wilt control has been the development of resistant varieties. Over 26 Fusarium wilt resistant vegetable varieties have been developed through plant breeding efforts (Mace, et. al., 1981). Asparagus is not considered to be isogenic and genetic variability found within the species and availability of germplasm from wild species should produce some genetic resistance within the species (Tiedgens, 1924, 1926; Robb, 1984). However, efforts world wide for the past 20 years of breeding have not produced any truly resistant varieties (Lougheed and Tiessen, 1985).

## Allelopathy

Plant residues from various sources contribute to the organic matter component of the soil. These tissues from young, mature and dead plant parts are ultimately decomposed in the soil by chemical or microbial breakdown. Thus, chemical components previously bound in the plant materials are made available for microbial and plant uptake. At any one time, the soil may contain a vast array of chemical components which may affect plant growth in either beneficial or detrimental ways.

Decomposition of plant residues in the soil associated with the formation of phytotoxic substances has been widely documented (Borner, 1960; McCalla and Haskins, 1961; Patrick and Koch, 1963; Toussoun and Patrick, 1963; Schroth and Hilderbrand, 1964; Toussoun, et. al., 1968; Lindermann, 1970; Patrick, 1971; Putnam and Duke, 1978; Stowe, 1979; Rice, 1979, 1984). In the early 1900's, DeCandolle (1932) had suggested that 'soil sickness' was due to crop plant exudates. In the past 25 years, a large amount of scientific effort has been devoted to elucidating how root exudates, leachates from living plants, and plant residues affect plant growth, interference between plant populations and microbial populations. This biochemical interaction that plants exert on other plants is called allelopathy. Allelopathy was first defined as any chemical effect of one plant upon another plant (Molisch, 1937). Allelopathy can be separated from other forms of plant interference in that the detrimental effects are exerted through

the release of a chemical into the environment (Muller, 1969; Fuerst and Putnam, 1983; Rovira, 1969; Whittaker and Feeny, 1971). Allelochemical stresses upon crop plants can reduce crop emergence, growth and yield, as well as resistance to plant pathogens (McCalla and Haskins, 1970; Linderman 1970; Bell, 1974). The science of allelopathy has recently been the focus of several books (Rice, 1984; Thompson, 1985; Putnam and Tang, 1986).

## Allelopathy and Microorganisms

Allelopathic effects appear to be especially important in natural communities dominated by a single species (Whittaker, 1975). However, relatively few reports have been published that examine how allelopathic compounds may affect microorganism, particularly plant pathogens. Unidentified fungitoxic exudates on leaf surfaces are believed to offer limited protection against plant pathogens (Barbosa and Saunders, 1986).

In an examination of the peach tree decline and replant problem, Chandler and Daniell (1974) found peach seedlings grown in either old peach soil or in the presence of peach soil leachates were more susceptible to infection by Pseudomonas syringae than seedlings grown in control soils or soils from a pecan orchard. They postulated that toxins from dead peach roots may predispose new trees to bacterial canker and thus contribute to peach decline. Amygdalin, a cyanogenic glycoside, was isolated from peach roots and believed to be the primary source of toxic substances present in the soil (Patrick, 1955). In the presence of enzymes provided by the microbial population in the soil, amygdalin is cleaved in two places producing two toxins, hydrogen

cyanide and benzaldehyde. He demonstrated that young peach roots are extremely susceptible to damage by these compounds while other Prunus spp. are less affected.

Exposure of tobacco plants to leachates obtained from decomposing rye and timothy residues increased the susceptibility of both resistant and susceptible varieties of tobacco to black root rot caused by Thielaviopsis basicola (Beck and Br.) Ferraris (Patrick and Koch, 1963). Using 16 different tobacco varieties ranging from susceptible to resistant to black root rot and 6 different isolates of T. basicola, they showed that exposure to the leachates overcame resistance of the tobacco to the pathogens. They postulated that the leachates were damaging the tobacco roots and, therefore, making the plants more suseptible for infection and colonization by the pathogen. They hypothesized toxins produced by rye or timothy were responsible for the breakdown of resistance seen in fields since rye and timothy were often used in rotation with tobacco. They concluded these toxins may be an important predisposition factor in the disease syndrome.

Toxic substances produced from decomposing residues of rye, barley, broccoli, and broad bean greatly enhanced the pathogenesis of F. solani (Mart.) Sacc. f.sp. phaseoli (Burk) Snyder and Hansen on beans (Toussoun 1963). Disease enhancement, measured by lesion development on bean stems, was greatest using extracts obtained during the early stages (less than 1 month) of decay of the residues. They postulated this enhancement was due to an additive effect of the extract and the pathogen; the extract was preconditioning the roots to fungal invasion. They noted that root rots are not necessarily caused by specific pathogens and hypothesized that organisms ordinarily causing little damage might become more pathogenic if conditions were

favorable for pathogen development. Other experiments showed these toxins had a direct effect on the host cells, altering the cell permeability. They concluded the resulting increased exudation of ninhydrin-positive compounds and other substances were readily available to organisms in the infection court and were mainly responsible for predisposing the host to infection by pathogenic organisms.

## Asparagus as an Allelopathic Plant

Recent investigations of the causal factors of asparagus decline have suggested that allelopathic compounds are present in asparagus root tissue (Laufer and Garrison, 1977; Shafer and Garrison, 1980a, 1980b, 1986; Yang, 1982, 1985; Young, 1984; Hartung and Stephens, 1983, 1984; Hodupp, 1983; Hartung and Putnam, 1985). Several studies have shown that allelochemicals released from both the growing asparagus plant and from the senescing root tissue are not only allelopathic to other plant species but are also autoallelopathic or autotoxic (Laufer and Garrison, 1977; Shafer and Garrison, 1980a, 1986; Yang, 1982, 1985; Hartung and Stephens, 1983; Hartung and Putnam, 1985). Water extracts from asparagus seedlings grown from tissue cultured plants inhibited the growth of asparagus seedlings grown in plastic pouches (Yang, 1982). Also, evidence shows that asparagus tissues incorporated into soil increase the incidence of asparagus root rot on asparagus seedlings (Hartung and Stephens, 1983). In the presence of dried asparagus root tissue incorporated into soil with or without either of the Fusarium pathogens, asparagus seedlings had more root rot than seen on control plants. The dry weight of those treatments was also less

than those of control plants. The Fusarium pathogens, in contrast to other soil-borne microorganisms, appeared insensitive to the allelopathic substances in the root and fern tissue (Hartung, 1983). Shafer reported that toxicity from asparagus root tissue decreased in a soil medium over time but made no attempt to determine if degradation was primarily through microorganisms (Shafer and Garrison, 1986). Also, they did not examine any effects from fern tissue.

In studies utilizing an XAD-4 resin trapping procedure originally developed to recover phenolic and chlorophenoxy herbicides from soil, compounds inhibitory to asparagus seedlings were collected from healthy asparagus plants grown in sand over a period of 6 months (Young, 1984). No differences were noted between 3 varieties of asparagus. In another report, he found that asparagus seedlings planted in unsterilized "used" vermiculite and treatments with root tissues alone incorporated into vermiculite, started to "yellow and wilt" after 21 days but were normal in controls and treatments with sterilized vermiculite and root tissues (Young, 1986). He postulated that there is an interaction between the residues and microorganisms. However, the exact nature of the "used vermiculite" or its microbial content was not published, making it difficult to assess the results.

## Isolated and Identified Compounds from Asparagus Tissue

Several compounds that may contribute to the autotoxic properties of asparagus have already been identified. Asparagusic acid, a 1,2-dithiolane, dihydroasparagusic acid and s-acetyldihydroasparagusic acid were isolated and identified from etiolated asparagus tissues by a

Japanese group in 1972 (Kitahara, et. al, 1972; Yanagawa, et. al.,1972; Yanagawa, et. al, 1973a). Asparagusic acid inhibited lettuce root and hypocotyl growth at 6.67 x $10^{-4}$ M, and was active on rice, rape, radish, carrot, and barnyardgrass at similar concentrations. It's activity closly parallelled that of abscisic acid. The structure and activity of asparagusic acid was confirmed by synthesis (Yanagawa, et. al., 1973b; Yanagawa, 1979). Dihydroasparagusic acid and S-acetyldihydroasparagusic acid were also isolated and their structures confirmed by synthesis (Yanagawa, 1973a, 1973c). They are reported to be inhibitory at the same concentration as asparagusic acid. In one report, these compounds were compared in activity to lipoic acid which is known to participate in the transfer of acyl groups. Since the asparagus compounds were similar to lipoid acid in structure, they examined if lipoic acid could be replaced with asparagusic acid or its derivatives (Yanagawa, et. al., 1973c). They used Streptococcus faecalis 10C1 since its growth is stimulated by lipoic acid. Asparagusic acid did increase growth of the bacteria and also stimulated the rate of pyruvate oxidation but at concentrations 10,000 times that of lipoic acid. They also examined the effects of asparagusic acids on the stimulation of pyruvate oxidation in asparagus mitochondria as compared to lipoic acid (Yanagawa, 1973d). Both asparagusic acid and dihydroasparagusic acid stimulated pyruvate oxidation but lipoic acid showed no effect. These effects were opposite those obtained with S. faecalis 10C1. Asparagusic acid is biosynthesized by a different pathway that that of lipoic acid (Perry, et. al. 1982). Other research has shown that dihydroasparagusic acid promoted rooting of mung bean cuttings at low concentrations ($10^{-9}$ -$10^{-5}$), and killed roots at higher concentrations ($10^{-4}$) (Kuhnle, et.

al., 1975a). This group also reported that dihdroasparagusic acid retarded germination of Pisum sativum cv. Early Alaska, but no effect from the compound was noted when plants were grown to first anthesis from seeds (Kuhnle, et. al. 1975b).

Asparagusic acid has also been isolated from the roots of asparagus and found to be inhibitory to nematodes, specifically the emergence of the second stage larvae of Heterodera rostochiensis and H. glycines, and the second stage larvae of H. rostochiensis and Meloidogyne hapla, and the larvae and adults of Pratylenchus penetrans and Pratylenchus curvitatus (Takasugi, et. al.,1975).

Young has reported that several phenolic compounds are released from the root system of intact asparagus plants (Young, 1986). Using an XAD-4 resin column (originally developed to recover phenolic and chlorophenoxy herbicides) attached to pots of sand where asparagus was growing, he isolated 3,4-dihydroxybenzoic acid, 2,6-dimethoxyacetophenone, and ß-(m-hydroxyphenyl)propionic acid. However, he did not report if these compounds were inhibitory to asparagus seedlings.

Though compounds isolated by the Japanese workers are considerably toxic to germination and radical elongation of several species, these compounds occur at extremely low concentrations in the tissues. Since asparagus possesses large storage roots that are continually dying as the crown grows, large amounts of asparagus root material may be present in asparagus fields at any one time. At this point, no one has isolated a compound or compounds that can reasonably account for all the inhibitory activity present in asparagus root tissue. Even though several compounds have been isolated from asparagus tissues or

artificial growth media, none have been shown to be active in the asparagus rhizosphere. In addition, no detailed biochemical work has been initiated to determine if components in root extracts affect specific sites in the asparagus tissue. Also, even though some research has concentrated on how asparagus tissues are degraded in the soil, little attention has been devoted to the fate of these components in natural soil and how microorganisms may be affecting the toxic substances present in the tissues.

In addition, there is also a need to further investigate how toxic components of the tissues may be affecting microbial populations in the soil. If asparagus extracts are inhibitory to the general microbial population and not to the Fusarium pathogens, this may decrease competition among microorganisms in the soil, thereby giving the root pathogens a competitive edge in the agroecosystem where asparagus is grown. It is the purpose of this dissertation to examine these questions, and to more fully elucidate the role of allelopathy in the asparagus decline syndrome.

## LITERATURE CITED


1. Alexander, M. 1961. Introduction to soil microbiology. John Wiley and Sons, Inc. NY. pp.
2. Anonymous. 1977. Michigan Asparagus Survey, Michigan Crop Reporting Service.
3. Anonymous. 1980. Michigan Agricultural Statistics. Michigan Dept. of Agriculture.
4. Barbosa, P., and J. A. Saunders. 1986. Plant Allelochemicals: Linkages between herbivores and their natural enemies. Recent Advances Phytochem. 19:107-137.
5. Booth, C. 1971. The Genus Fusarium. Commonwealth Mycological Institute, Kew, Surrey, England. pp. 237.
6. Borner, H. 1955. Untersuchungen uber Phenolische verbindungen aus Gefreidstroh and Getreiderukstanden. Naturwissenschaften. 42:583-584.
7. Chandler, W. A., and J. W. Daniell. 1974. Effect of leachates from peach soil and roots on bacterial canker and growth of peach seedlings. Phytopathol. 64:1281-1284.
8. Cohen, S. I., and Heald, 1941. A wilt and root rot of asparagus caused by Fusarium oxysporum Schlecht. PDR. 25:503-509.
9. Damicone, J. P., and D. R. Cooley. 1981. Benomyl in acetone eradicates Fusarium moniliforme and F. oxysporum from asparagus seed. Plant Disease. 65:892-893.

10. Damicone, J. P., and W. J. Manning. 1984. Frequency and pathogenicity of Fusarium spp. isolated from first-year asparagus grown from transplants. Plant Disease. 69:413-416.

11. DeCandolle, M. A. P. 1832. "Physiologie Vegetale," Tome III. Bechet Jeune. Lib. Fac. Med. Paris. pp. 1474-1475.

12. Endo, R. M., and E. C. Burkholder. 1971. The association of Fusarium moniliforme with the crown rot complex of asparagus. Phytopathol. 99:122-125.

13. Evans, T. A. 1985. Asparagus viruses and their contributions to Michigan Asparagus Decline. PhD. Thesis. Michigan State University. pp.

14. Evans, T. A., and C. T. Stephens. 1985. Contributions of asparagus viruses to Michigan Asparagus Decline. pp. 207-219 In: Proc. of the Sixth International Symposium on Asparagus. E. C. Lougheed and H. Tiessen, eds., Univ. Guelph. pp.408.

15. Evans, T. A., G. Safir, and C. T. Stephens. 1985. Vesicular-Arbuscular mycorrhizal fungi and their importance in Asparagus Decline. p.399. In: Sixth International Symposium on Asparagus. E. C. Lougheed and H. Tiessen, eds., Univ. Guelph. pp. 408.

16. Farrish, L. R. 1937. Fall cuttings of asparagus compared with spring cuttings under Mississippi conditions. Proc. Am. Soc. Hort. Sci. 35:693-695.

17. Fuerst, E. P. and A. R. Putnam. 1983. Separating the competitive and allelopathic components of interference: Theoretical principles. J. Chem. Ecol. 9:937-944.

18. Grogan, R. G., and K. A. Kimble. 1959. The association of Fusarium wilt with the asparagus decline and replant problem in California. Phytopathol. 99:122-125.

19. Hanna, G. C. 1947. Asparagus production in California. Calif. Agr. Ext. Serv. Circ. 91. pp 23.

20. Hartung, A. C. 1983. Effects of allelopathic substances produced by asparagus on the incidence and severity of Fusarium crown rot. Thesis for the M.S. Degree. Michigan State University. 65 pp.

21. Hartung, A. C., and C. T. Stephens. 1984. Allelopathic properties of asparagus: Interaction with Fusarium spp. and bioassay techniques. Phytopathol. 74 (7):800 (Abstr.)

22. Hartung, A. C., and C. T. Stephens. 1983. Effects of allelopathic substances produced by asparagus on the incidence and severity of Fusarium crown rot. J. Chem. Ecol. 9:1163-1174.

23. Hartung, A. C., and A. R. Putnam. 1985. Extracts of asparagus root tissues are phytotoxic. Proc. of the Sixth International Asparagus Symposium. E. C. Lougheed and H. Tiessen, eds., University of Guelph. pp. 258-266.

24. Hendrix, F. F., Jr., and L. E. Nielson. 1958. Invasion and infection of crops other than the forma suscept by Fusarium oxysporum f. batatas and other formae. Phytopathol. 48:224-228.

25. Herner, R. C., and G. Vest. 1974. Asparagus Workshop Proceedings. Department of Horticulture, Michigan State University, E. Lansing, MI pp. 79.

26. Hodupp, R. M. 1983. Investigation of factors which contribute to asparagus (Asparagus officinalis L.) decline in Michigan. Thesis for the M.S. Degree. Michigan State University. pp. 53.

27. Johnston, S. A., J. K. Springer, and G. D. Gewis. 1979. Fusarium moniliforme as a cause of stem and crown rot of asparagus and its association with asparagus decline. Phytopathol. 69:778-780.

28. Kitihara, Y., H. Yanagawa, T. Kato, and N. Takahashi. 1972. Asparagusic acid, a new plant growth inhibitor in etiolated young asparagus shoots. Plant and Cell Physiol. 13:923-925.

29. Kuhnle, J. A., J. Corse, and B. G. Chan. 1975a. Effect of dihydroasparagusic acid on the germination of Pisum sativum cv. Early Alaska. Biochem. Physiol. Pflanzen (BPP). Bd. 167:S. 188-190.

30. _____ 1975b. Promotion of rooting of mung bean cuttings by dihydroasparagusic acid synergistic interaction with Indoleacetic acid. Biochem. Physiol. Pflanzen (BPP), Bd. 167, S. 553-556.

31. Lacy, M. L. 1979. Effects of chemicals in stand establishment and yields of asparagus. PDR. 63:612-616.

32. Laufer, G. A., and S. A. Garrison. 1977. The effect of asparagus tissue on seed germination and asparagus seedling growth. Possible allelopathic interaction. HortSci. 12:385. (Abstr.).

33. Linderman, R. G. 1970. Plant residue decomposition products and their effects on host roots and fungi pathogenic to roots. Phytopathol. 60:19-22.

34. Lougheed, E. C., and H. Tiessen. 1985. Proceedings of the Sixth International Asparagus Symposium. University of Guelph. Guelph, Pp.408.

35. Mace, M. E., A. A. Belland, and C. H. Beckman. 1981. Fungal wilt disease of Plants. Academic Press. NY. Pp 639.

36. Molot, P., and J. Simone. 1965. Study of two factors which aid contamination of asparagus. C. R. Hebd. Seances Acad. Agr. France. 51(5):314-317.

37. Manning, N. J., and P. M. Vardaro. 1977. Soil fumigation and preplant fungicide crown soaks effects in plant growth and Fusarium incidence in newly planted asparagus. PDR 61:355-357.

38. McCalla, T. M., and F. A. Haskins. 1964. Phytotoxic substances from soil microorganisms and residues. Bacteriol. Rev. 28:181-207.

39. Molisch, H. 1937. Der Einfluss einer pflanze auf die audere allelopathie. Jena:Fisher.

40. Muller, C. H. 1969. Allelopathy as a factor in ecological processes. Vegetatio. 18:348-357.

41. Nelson, P. E., T. A. Toussoun, and R. J. Cook. 1981. Fusarium: Diseases, Biology, and Taxonomy. Penn. St. Univ. Press. pp. 457.

42. Nelson, P. E., G. H. Coon, and L. C. Cochran. 1937. Fusarium yellows disease of celery. Mich. ag. Exp. Sta. Tech. Bull. 155:1-74.

43. Nyvall, R. F., and T. Kommedahl. 1968. Individual thickened hyphae as survival structures of Fusarium moniliforme in corn. Phytopathol. 58:704-707.

44. Parry, R. J., A. E. Mizusawa, and M. Ricciardone. 1982. Biosynthesis of sulfur conpounds. Investigations of the biosynthesis of asparagusic asid. J. Am. Chem. Soc. 104:142-143.

45. Patrick, Z. A., T. A. Toussoun, and W. C. Snyder. 1963. Phytotoxic substances in arable soils associated with decomposition of plant residues. Phytopathol. 53:152-161.

46. Patick, Z. A., and L. W. Koch. 1963. The adverse influence of phytotoxic substances from decomposing plant residues on resistance of tobacco to black root rot. Can. J. Bot. 41:747-758.

47. Patrick, Z. A. 1971. Phytotoxic substances associated with the decomposition in soil of plant residues. Soil Sci. 111:13-18.

48. Penn, D. J. and J. M. Lynch. 1982. The effect of bacterial fermentation of couchgrass rhizomes and Fusarium culmonoum on the growth of barley seedlings. Plant Pathol. 31:39-43.

49. Putnam, A. R. and W. B. Duke. 1978. Allelopathy in agroecosystems. Ann. Rev. Phytopathol. 16:431-451.

50. Putnam, A. R., and C-S. Tang. 1986. The Science of Allelopathy. John Wiley, and Sons, Inc. NY pp.317.

51. Rice, E. L. 1984. Allelopathy. Academic Press, Inc. Pp.422.

52. Rice, E. L. 1979. Allelopathy-An Update. Bot. Rev. 45:15-109.

53. Robb, A.R., 1984. Physiology of asparagus (Asparagus officinalis) as related to the production of the crop. New Zealand J. Exp. Agri. 12:251-260.

54. Rovira, A. D. 1969. Plant root exudates. Bot Rev. 35:35-39.

55. Shafer, W. E., and S. A. Garrison. 1980a. Effects of decomposing asparagus root tissues on lettuce, tomato, and asparagus seed emergence. HortSci. 15:406 (Abstr.)

56. Shafer, W. E., and S. A. Garrison. 1980b. Effects of asparagus root extracts on lettuce and asparagus seed germination and growth. HortSci. 15: 406-408 (Abst.)

57. Shafer, W. E., and S. A. Garrison. 1986. Allelopathic effects of soil incorporated asparagus roots on lettuce, tomato, and asparagus seedling emergence. HortSci. 21(1):82-84.

58. Shroth, M. N., and D. C. Hildebrand. 1964. Influence of plant exudates on root infecting fungi. Annual. Rev. Phytopathol. 2:101-132.

59. Stowe, L. G. 1979. Allelopathy and its influence on the distribution of plants in an Illinois old-field. J. Ecol. 67:1065-085.

60. Takasugi, M., Y. Yachida, M. Anetai, T. Masamune, and K. Kegasawa. 1975. Identification of asparagusic acid as a nematicide occurring naturally in the roots of asparagus. Chem. Lett. Pp. 43-44.

61. Takatori, F., and F. Souther. 1978. Asparagus Workshop Proceedings. Department of Plant Sciences, Univ. Of Calif. Riverside. Pp. 100.

62. Takatori, F., J. I. Stillman, and F. D. Souther. 1970. Asparagus yields and plant vigour as influenced by time and duration of cutting. Calif. Agri. 24(2):10-12.

63. _____1974. Influence of planting depth on production of green asparagus. Calif. Agri. 28(1):4-5.

64. Tiedjens, V. A. 1924. Some physiological aspects of Asparagus officinalis. Proc. Am. Soc. Hort. Sci. 21:129-140.

65. Tiedjens, V. A. 1926. Some observations on root and crown bud formation in Asparagus officinalis. Proc. Am. Soc. Hort. Sci. 23:189-195.

66. Thompson, A. C. 1985. The Chemistry of Allelopathy. Biochemical Interactions Among Plants. ACS Symposium Series 268. Am. Chem. Soc. pp.470.

67. Toussoun, T. A. and Patrick, Z. A. 1963. Effect of phytotoxic substances from decomposing plant residues on root rot of bean. Phytopathol. 53:265-270.

68. Toussoun, T. A., A. R. Weinhold, R. G. Linderman, and Z. A. Patrick. 1968. Nature of phytotoxic substances produced during plant residue decomposition in soil. Phytopathol. 58:41-45.

69. VanBakel, J. M. M., and J. J. A. Kerstems. 1970. Root rot in asparagus caused by Fusarium oxysporom f. sp. asparagi. Neth. J. Plant. Path. 76:320-325.

70. Walker, J. C. 1952. Diseases of Vegetable Crops. McGraw-Hill Book Co. NY.

71. Whittaker, R. H. 1975. Communities and Ecosystems, 2nd ed. MacMillan Pub., Inc. NY. pp.358.

72. Whittaker, R. H. and P. Feeny. 1971. Allelochemicals: chemical interaction between species. Science. 171:757-770

73. Woltz, S. S., and J. P. Jones. 1981. Nutritional requirements of Fusarium oxysporum: Basis for a disease control systen. In: Fusarium: Disease, Biology, and Taxonomy. P. E. Nelson, T. A. Toussoun, R. J. Cook, eds., Penn. State Univ. Press. pp 457.

74. Yanagawa, H. 1979. Preparation and determination of asparagusic acid. In: Methods in Enzymology. 62:181-184.

75. Yanagawa, H., T. Kato, Y. Kitahara. 1972. Asparagusic acid, dihydroasparagusic acid and s-acetyldihydroasparagusic acid, a new plant growth inhibitors in etiolated young Asparagus officinalis. Tetrahedron Letters 25:2549-2552.

76. _____ 1973a. Asparagusic acid-s-oxides, new plant growth regulators in etiolated young asparagus shoots. 1973. Tetrahedron Letters. 13:1073-1075.

77. Yanagawa, H. T. Kato, H. Sagami, and Y. Kitihara. 1973b. A convenient procedure for the synthesis of asparagusic acids. Synthesis. 607-608.

78. Yanagawa, H., T. Kato, Y. Kitahara, N. Takahashi. 1973c. Stimulation of growth and pyruvate oxidation in Streptococcus faecalis by asparagusic acid and its derivatives. Plant and Cell Physiol. 14:791-795.

79. Yanagawa, H., T. Kato, and Y. Kitahara. 1973. Stimulation of pyruvate oxidation in asparagus mitochondria by asparagusic acids. Plant and Cell Physiol. 14:1213-1216.

80. Yang, Hsu-Jen. 1982. Autotoxicity of Asparagus officinalis L. J. Am. Soc. Hort. Sci. 107:860-862.

81. Yang, Hsu-Jen. 1985. Autotoxic and allelopathic characteristics of Asparagus officinalis L. Proc. of the Sixth International Asparagus Symposium. E. C. Lougheed and H. Tiessen, eds., University of Guelph. pp. 267-276.

82. Young, C. C. 1984. Autotoxication in root exudates of Asparagus officinalis L. Plant and Soil. 82:247-253.

83. Young, C. C. 1986. Autotoxication of Asparagus officinalis L. in: The Science of Allelopathy. A. R. Putnam and Chung-Shih Tang, eds., John Wiley and Sons, Inc. NY. pp. 317.

# CHAPTER II

## ALLELOPATHIC POTENTIAL OF ASPARAGUS RESIDUES IN STERILE AND NONSTERILE SOIL


## ABSTRACT

Asparagus, (Asparagus officinalis L.) has been reported to have allelopathic potential and compounds released from its tissues were hypothesized to increase Fusarium root rot on asparagus seedlings. Dried asparagus root and fern tissues were incorporated into sterile or nonsterile soil to determine if microbial populations influenced their inhibitory activities. Four different indicator species; Lepidium sativum, cv. Curly Cress, Lactuca sativum, cv. Grand Rapids, Digitaria ischaemum, and Echinochloa crusgalli were utilized in bioassays. Species responded differently to compounds released from the root and fern tissues when grown under aerobic or anaerobic conditions, as well as sterile or nonsterile soil conditions. Both tissues were inhibitory to seedling development under both aerobic and anaerobic conditions. Fern tissue was less inhibitory than root tissue to seedling root and shoot growth. Microbial populations in the soil generally degraded tissue toxicity, but in one case slightly increased its inhibitory activity for a short time. Purified extracts of asparagus root tissues were inhibitory to nine of ten indicator species. Data from purified extract experiments agreed with data from soil bioassays.

## INTRODUCTION

In the past eight years several research papers have reported asparagus (Asparagus officinalis L.) to be an allelopathic plant (Laufer and Garrison, 1977; Shafer and Garrison, 1980a; 1980b; 1986; Yang, 1982; Hartung and Stephens, 1984; Hartung and Putnam, 1985; Young, 1986). Allelopathy has been defined as the detrimental influence of one plant on another through the release of chemicals into the environment (Molisch, 1937). Chemical interference by compounds released from plants has been identified as an important component of plant interference in natural and agroecosystems (Borner, 1950; Tukey, 1969; Rice, 1984, 1979; Putnam and Duke, 1978;). Allelochemical stresses upon crop plants can reduce crop emergence, growth and yield, as well as resistance to plant pathogens (Bell, 1974; Linderman, 1970; McCalla and Haskins, 1970.). In the case of asparagus, not only are these compounds thought to be detrimental to other plants, but are also considered to be autotoxic or autoallelopathic (Yang, 1982; Hartung and Putnam, 1985; Shafer, 1980b; Young, 1984). Shafer (1986) reported that toxicity from asparagus root tissue decreased in a soil medium over time but made no attempt to determine whether microorganisms may have degraded the compounds released from the root tisssue.

When asparagus was planted in soil amended with sterilized dried root or rhizome tissue in the presence of Fusarium oxysporum (Schlecht.) Snyd. and Hans. f.sp. asparagi Cohen or F. moniliforme Sheldon Snyd. and Hans., there was a significant increase in the

severity of root rot caused by these two pathogens and a decrease in the dry weight of the asparagus crown (Hartung and Stephens, 1984). These data suggest that chemical components released directly from the root tissue or altered by microorganisms were either affecting the asparagus plant directly or interacting with microflora of the rhizosphere to increase the susceptibility of the asparagus plant to infection by the disease organisms. Therefore, this research was done to determine the fate of the compounds released from the asparagus root tissue in the soil, to determine the influence of soil microorganisms on the toxicity of the root and fern tissue under both aerobic and anaerobic conditions, and to assess the effects of purified asparagus root extracts on seed germination and radical growth of different seedling species.

## MATERIALS AND METHODS

**Preparation of Asparagus plant material.** A commercial asparagus field (<20 yr old) in Oceana County, MI was excavated and asparagus crowns (cv. Martha Washington) were collected. The storage and fibrous roots were separated from the rhizomes and washed after which all dead and visably diseased plant material was discarded. The roots were oven dried at 40 C and ground in a Wiley Mill (mesh screen size 1 mm). Fern tissue was clipped from the crowns and prepared in the same manner as the root tissue. All plant tissues were sterilized with propylene oxide gas (Tuite, 1969). Containers with sterilized tissue were allowed to exhaust under a fume hood for 24 hr to dissipate all propylene dioxide. Dried tissues were stored in brown glass bottles at -20 C until needed for petri plate bioassays or extractions.

**Assessment of microbial contamination of asparagus tissues.** To ascertain that sterilized tissues were free from Fusarium sp., or other contaminating microorganisms, 1 g of dried tissue was spread evenly over the surface of a 9 cm petri plate containing Komada's medium (Komada, 1975), potato dextrose agar (PDA) (39 g PDA, Difco Laboratories, Detroit, MI, in 1 liter distilled water ($DH_2O$)). There were 4 replica plates per tissue. After one week, plates were assessed for the number of fungal colonies growing on the medium. Plates were examined every day for contaminating organisms for ten days.

**Toxicity of asparagus tissues in soil.** Spinks sandy loam soil (Psammentic Hapludalf, sandy, mixed, mesic, with 1% organic matter, pH 6.5) collected from a non-agricultural site, was air dried, sifted through a 0.5 mm screen, weighed into 150 g samples and either tinsdillated (one hr each time at 120 C; 23 psi) or left nonsterilized. Sterility of the soil was confirmed by randomly selecting five different soil samples and plating out two dilutions/soil sample on PDA and observing for microbial growth over a period of 14 days.

In order to evaluate the fate of the toxic substances present in asparagus tissues when exposed to microorganisms, tissues were incorporated into sterile and nonsterile soil and exposed to anaerobic and aerobic conditions. To assess the initial tissue toxicity, a "non-incubated treatment" was done for each set of experiments. Tissues were incorporated into the soil and indicator species placed immediately on the soil surface. Asparagus root or fern tissue previously sterilized using propylene oxide gas was then mixed thoroughly with the soil samples at 0.5%, 1% and 2% by weight and poured into square petri plates (10 x 10 x 1.5 cm). Sterile $DH_2O$ was

added to each plate at a water potential of -0.01 Bars for each treatment. Paper toweling cut into 1 cm fragments was also incorporated into the soil at similar rates to serve as a control for physical influence of the tissues. Filter papers (Whatman #1) were cut to cover one half of the surface of the soil and ten seeds each of two indicator species were placed on the filter papers. The plates were placed vertically in a moist chamber (to induce geotrophic growth) for 72 hr then root and/or shoot length or percent germination were measured for each indicator species.

To ascertain the effects of microorganisms under aerobic and anaerobic conditions, two more treatments were included in the experiment. A similar group of the plates not seeded with the indicator species was incubated in a moist chamber for 72 hr at 27 C in the dark and designated the "aerobic treatment". The remaining plates without the indicator species were placed in a desiccation chamber and flushed repeatedly with nitrogen to assure all oxygen was removed from the chamber. The plates were incubated under anaerobic conditions for 72 hr in the dark. This group was designated the "anaerobic treatment". Both the aerobic and anaerobic treatments were then planted as before with the same two seed species as the nonincubated treatment, bioassayed and measured as above (Table 1). Seedling species used as indicator species were lettuce, Lactuca sativa L. cv. Grand Rapids, Lepidium sativum L. cv. Curly Cress, smooth crabgrass, Digitaria ischaemum (Schreb.) Muhl., and barnyardgrass, Echinochloa crusgalli (L.) Beauv. These species were chosen for uniformity of germination rates, and as representatives of both monocot and dicot plants. There were three replications for each treatment, and the data were subjected to an analysis of variance and trend analysis. The

Table 1. Experimental factors for soil bioassays of the response of indicator seedling species to dried asparagus root or fern tissues incorporated into sterile or nonsterile soil. The design was a randomized complete block with 3 replications.

| Incubation | Soil Condition | Tissue | Level (%) |
|---|---|---|---|
| Not Incubated | Sterile | Root | 0.0 |
| Anaerobic | Nonsterile | Fern | 0.5 |
| Aerobic | | | 1.0 |
| | | | 2.0 |

experiment was repeated twice and data presented is from one representative experiment.

**Preparation of purified asparagus root extracts.** Previously published data from several laboratories have indicated that water extracts of asparagus root tissues are inhibitory to seedling germination and radical elongation (Yang, 1982; Hartung and Stephens, 1984; Hartung and Putnam, 1985 Shafer and Garrison, 1986). Therefore water extracts from asparagus root tissue were subjected to chemical extraction to purify the chemical components implicated in the inhibitory responses. All fractions throughout the isolation procedure were bioassayed on curly cress to determine their relative biological activities. Ground asparagus root tissue was extracted by stirring overnight at 4 C (1:10 root tissue:$DH_2O$). The particulates were then removed by filtering through four layers of cheese cloth and centrifuging at 6,000 g for 20 min. The supernatant was decanted carefully from the pellet (the pellet was discarded) and then precipitated with acetone (4:1 v:v acetone:sample) overnight at 4 C. The precipitant was discarded and the liquid concentrated to 1/4 the original volume on a Buchi Rotary evaporator. This concentrate was extracted with chloroform 3 times (1:1 v:v). The chloroform fraction was reduced to dryness, weighed, redissolved in methanol, filter sterilized, and then bioassayed using curly cress as an indicator. For bioassay, the fraction was dissolved in methanol at known concentrations and applied to filter paper in 6 cm petri plates. The methanol was allowed to evaporate, then 1.0 ml of $DH_2O$ and 10 curly cress seeds were added to each plate. The chloroform fraction was further purified using an octadecyl bonded phase solid

support in a Baker flash chromatography column (190 mm x 20 mm) eluted with a step gradient of Acetonitrile 100% to Methanol 100% at 25% intermediate steps in 100 ml fractions. Pressure for the column was provided by laboratory airline at a rate of 0.2 cm/sec. Fractions (25 ml) from the column were collected, spotted on thin layer chromatography plates (TLC) (Whatman silica gel 60 F-254; mobile phase, chloroform:methanol, 9:1) then recombined according to similar Rf values when compared under TLC as above. The fractions were dried under nitrogen, weighed, and bioassayed. The inhibitory fractions were combined and separated further on a silica gel solid support in a Baker flash chromatography column (180 x 30 mm) using a gradient of chloroform 100% to methanol 100% in the following order: 100% chloroform, 98:2, 96:4, 90:10, 85:15, 80:20, 70:30, 40:60, 100% methanol. Each gradient step was applied to the column in 200 ml fractions. Air pressure for the column was as above. Fractions of 75 ml each were collected and developed on TLC plates as above. Fractions with similar Rf values were combined, dried under nitrogen, weighed and bioassayed on curly cress as above. One inhibitory fraction from the column was used in bioassays using all the seedling species.

**Bioassay of partially purified compounds on indicator seed species.** A preliminary bioassay was done with ten different seed species to determine the optimum time of germination and optimum number of seeds necessary to produce relevant data in the bioassay studies (Table 2). All seed species were bioassayed at concentrations of 150, 100 and 50 ppm.

For bioassays, the inhibitory fraction was resuspended in methanol, diluted to the proper concentration, then spotted on Whatman

Table 2: Percent germination and time of evaluation of indicator species used in petri plate bioassays with purified asparagus root extract from a silica gel "flash" column.

| Species | Estimated Germination(%)[a] | Incubation period | Seeds per Petri plate |
|---|---|---|---|
| Lactuca sativa L., cv Grand Rapids | 85 | 72 | 25 |
| Raphanus sativus L., cv Cherrybell | 100 | 72 | 20 |
| Amaranthus retroflexus L. | 40 | 72 | 50 |
| Lepidium sativum, cv curly cress | 100 | 72 | 20 |
| Portulaca oleracea L. | 90 | 96 | 25 |
| Lycopersicon esculentum, L. cv Lafayette | 85 | 96 | 25 |
| Setaria italica (L.) Beauv. | 85 | 72 | 20 |
| Digitaria sanguinalis (L.) Beauv. | 65 | 96 | 30 |
| D. ischaemum (Schreb.) Muhl. | 60 | 96 | 35 |
| Echinochloa crusgalli (L.) Beauv. | 40 | 96 | 50 |

[a]based on a sample of 100 seeds/species.

#1 filter paper in a 60 x 15 mm sterile petri dish. The methanol was allowed to evaporate from the filter paper, then 1.5 ml of $DH_2O$ was added to the plate. The seeds to be tested were then placed in the dishes and spread evenly around the plates with a glass rod. After the designated incubation period, seeds were evaluated for root and shoot length as well as percent germination. Solvent and $DH_2O$ controls were also included in each assay. Due to scarcity of isolated components, there were two replications per treatment. Data were subjected to Analysis of Varience and trend analysis if applicable. The solvent control was used as the zero level in all trend analysis.

## RESULTS AND DISCUSSION

**Assessment of microbial contamination of asparagus tissues.** After a period of ten days, no Fusarium spp. or other microbial colonies were present in asparagus tissue samples sterilized with propylene dioxide gas.

**Toxicity of asparagus tissues in soil.** Within each seedling species, germination and growth that occurred with paper toweling and the no-tissue controls were not significantly different. Therefore, results obtained were not attributed to the physical presence of tissue in soil. As expected, indicator species varied in their response to asparagus root and fern tissue when these tissues were incorporated into sterile and nonsterile soil. In almost all instances, the third order interactions of tissue level x tissue source x soil condition were significant. For this reason and to provide consistency in

presentation, the graphs showing these interactions are depicted throughout. For lettuce there was highly significant interaction for the linear influences of tissue level x tissue source x soil condition (Figure 1). There was also an interaction between treatment type and soil condition. Initially, root and fern tissues were equally inhibitory to root growth in the nonincubated treatment. However, root tissue showed decreased inhibitory activity under nonsterile conditions in both aerobic and anaerobic incubations as compared to the nonincubated treatments. Fern tissue was equally toxic to lettuce radical growth under all treatment regimes. These data indicate that lettuce seedlings were less susceptible to components released from the root tissue than to components present in the fern tissue.

For cress root growth, there was a highly significant interaction of incubation x soil condition x tissue level (Figure 2). Under all treatments, root and fern tissue under nonsterile soil conditions were equally toxic to cress root growth. Trend analysis revealed the response was primarily linear ($F=29.45$ for linear response). Under anaerobic and sterile conditions, the inhibitory activity of fern tissue was greater than root tissue except at the lowest level of incorporation of tissue into soil. As with lettuce, there was also a highly significant interaction between soil condition x tissue type x tissue level. Inhibitory activity from root tissue was apparently not affected by resident microbial populations in the nonsterile soil treatments. However, in all treatments, inhibitory activity of the fern tissue was significantly decreased when exposed to a nonsterile soil condition and this response was quadratic. Although lettuce and cress generally responded alike, they did respond differently to the different asparagus tissues incorporated into the soil. Cress was most

Figure 1: Response of Lettuce, cv. Grand Rapids to increasing concentrations of dried asparagus root or fern tissue in sterile or nonsterile soil incubated under aerobic or anaerobic conditions. Not incubated treatment served as a control for initial activity present in the tissues. Interaction of tissue levels x tissue type x soil condition; and treatment type x soil condition were significant at P=0.01.

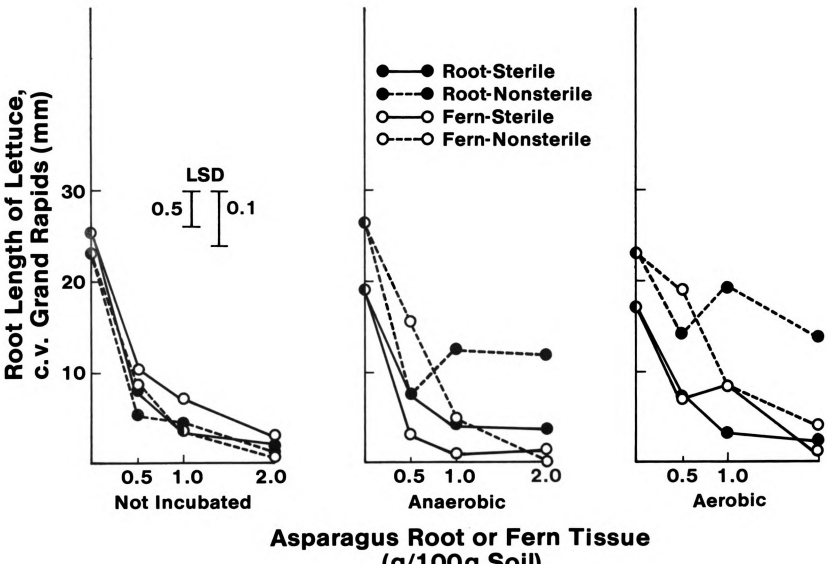
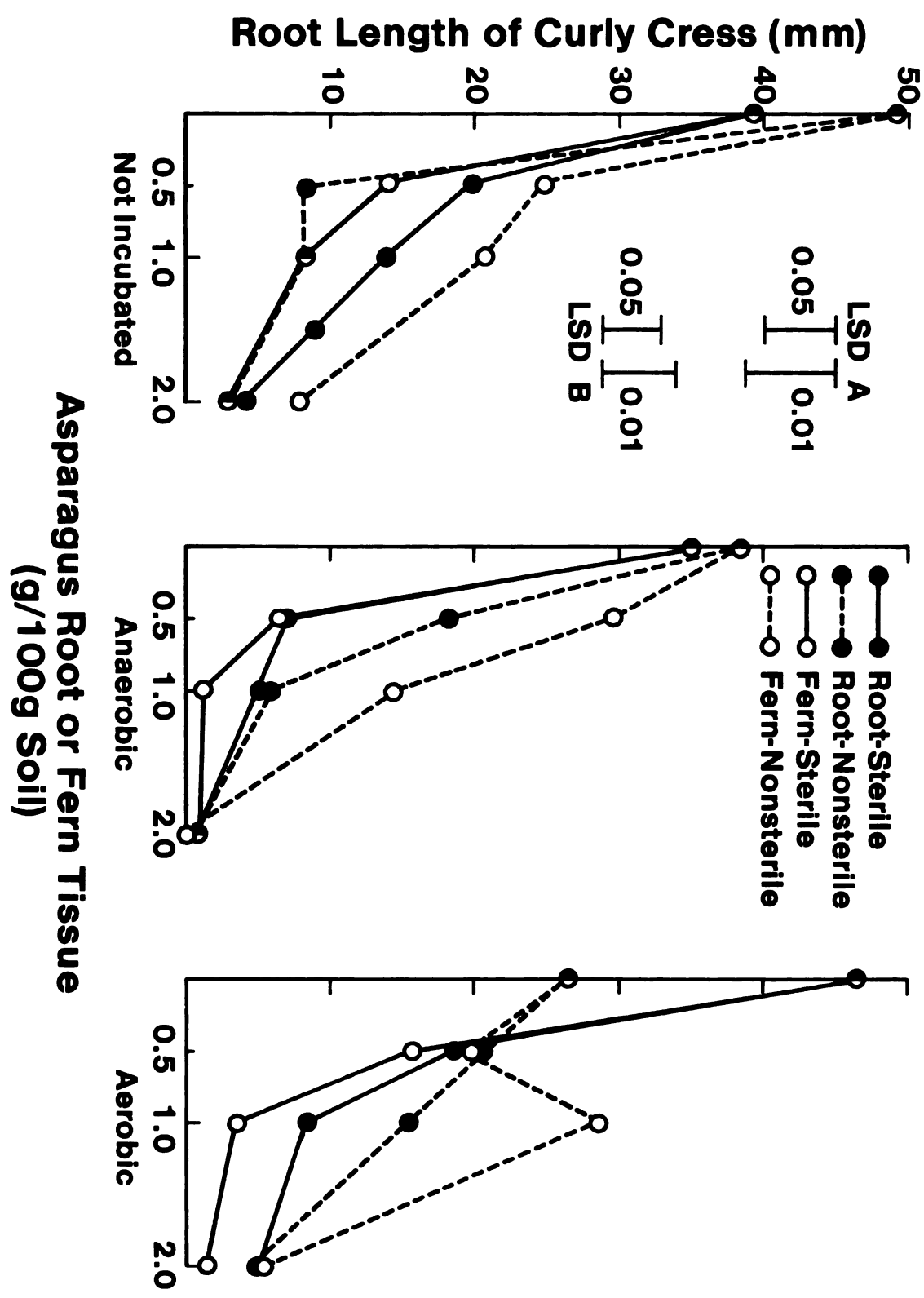

Root Length of Lettuce, c.v. Grand Rapids (mm)
30
20
10
LSD
0.5
0.1
Root-Sterile
Root-Nonsterile
Fern-Sterile
Fern-Nonsterile
0.5 1.0 2.0
Not Incubated
0.5 1.0 2.0
Anaerobic
0.5 1.0
Aerobic
Asparagus Root or Fern Tissue (g/100g Soil)

Figure 2: Response of Curly Cress root growth to increasing concentrations of dried asparagus root or fern tissue in sterile or nonsterile soil incubated under aerobic or anaerobic conditions. Interactions of incubation x tissue source x soil condition (LSD=A); and soil condition x tissue source x tissue level (LSD=B) were significant at P=0.01.


Root Length of Curly Cress (mm)
10
20
30
40
50
LSD A
0.05
0.01
LSD B
0.05
0.01
Root-Sterile
Root-Nonsterile
Fern-Sterile
Fern-Nonsterile
Not Incubated
Anaerobic
Aerobic
0.5
1.0
2.0
Asparagus Root or Fern Tissue
(g/100g Soil)

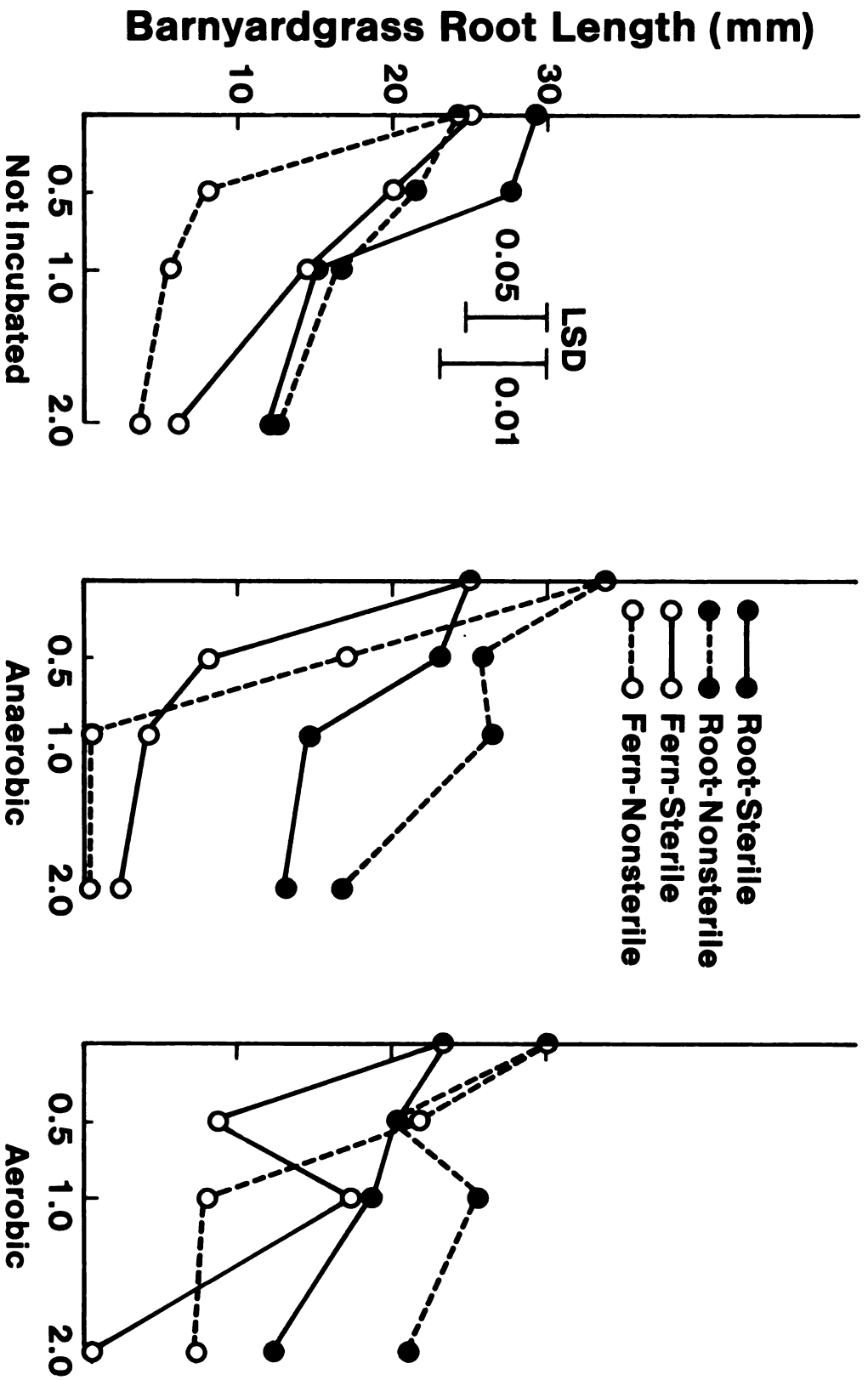
inhibited by root tissue, whereas lettuce was inhibited more by the fern tissue.

Cress shoot growth showed similar responses with the following exceptions (data not presented). Under anaerobic and nonsterile soil conditions, inhibitory activity from both root and fern tissue was greater than in aerobic or nonincubated controls. Under sterile conditions, fern tissue was more inhibitory than root tissue, while under nonsterile conditions root tissue was more inhibitory except at the highest concentrations of tissue incorporation. These data suggest that inhibitory compounds more active on shoot growth of cress may be degraded more quickly by the resident aerobic microbial population for fern tissue. However, compounds from root tissue are not degraded by the natural microbial populations.

For root growth of barnyardgrass, there was a significant interaction between tissue type x soil condition x tissue level (Figure 3). No other interactions showed significance. Root tissue showed less activity under nonsterile soil conditions in both the anaerobic and aerobic treatments. In the nonincubated treatment, fern tissue was more inhibitory under nonsterile soil conditions. The data indicate that compounds present in the fern tissue are more inhibitory to barnyardgrass root growth, and that inhibitory activity may be greater upon initial breakdown by microbes but is quickly degraded as time elapses. Shoot growth for barnyardgrass was also inhibited significantly by fern tissue but not by root tissue (data not shown).

Since a preliminary experiment indicated that germination and growth of smooth crabgrass seeds varied considerably more than other species, 100 seeds were used in bioassays with this indicator species.

Figure 3: Response of barnyardgrass root length to increasing concentrations of dried asparagus root or fern tissue in sterile or nonsterile soil incubated under aerobic or anaerobic conditions. Non-incubated treatments served as controls for the initial inhibitory activity present in the tissues. Interaction of tissue type x soil condition x tissue level were significant at P=0.01.


Barnyardgrass Root Length (mm)
10
20
30
0.05
LSD
0.01
Not Incubated
Anaerobic
Aerobic
0.5
1.0
2.0
Root-Sterile
Root-Nonsterile
Fern-Sterile
Fern-Nonsterile
Asparagus Root or Fern Tissue
(g/100g Soil)

Analysis of smooth crabgrass shoot growth showed significant interactions only between incubation type x tissue level, and soil condition x tissue level (Figure 4). There was no inhibitory activity from asparagus tissue on smooth crabgrass except at the higher levels of root tissue incorporation under anaerobic and sterile soil conditions or fern tissue under aerobic and sterile conditions. Therefore, compounds responsible for inhibition of shoot growth can be degraded by the microbial populations in the soil.

Germination of smooth crabgrass was also reduced, particularly with root tissue (Figure 5). In the nonincubated treatments, root and fern tissue were equally toxic to germination. Root tissue did not inhibit germination under either sterile or nonsterile soil conditions in the aerobic treatment, but was highly inhibitory under anaerobic conditions. Fern tissues remained only slightly inhibitory to germination in both sterile and nonsterile conditions, but did not inhibit germination greater than 50% under any treatment conditions. These data suggest that fern tissue is only marginally toxic to germination of smooth crabgrass and that toxicity is either unaffected by microbial breakdown in the soil, or that compounds responsible for the inhibitory response are at low concentrations in the asparagus fern tissues. Also, toxicity from root tissue, although more inhibitory initially when compared to fern tissue, is degraded by the microbial populations in the soil, and therefore loses its toxic properties.

Data from all these bioassays indicate differential response by plant species to toxic substances released into the soil from both root and fern tissues of the asparagus plant. Several reports have been published concerning toxicity from root tissue but little research has been done concerning activity from fern tissue. Fern residues did not

Figure 4: Response of Smooth Crabgrass shoot growth to increasing concentrations of dried asparagus root or fern tissue in sterile or nonsterile soil incubated under aerobic or anaerobic conditions. Not incubated plates served as controls for initial inhibitory activity present in the tissues. Interaction between incubation type x tissue level; and soil condition x tissue level were significant at P=0.05.

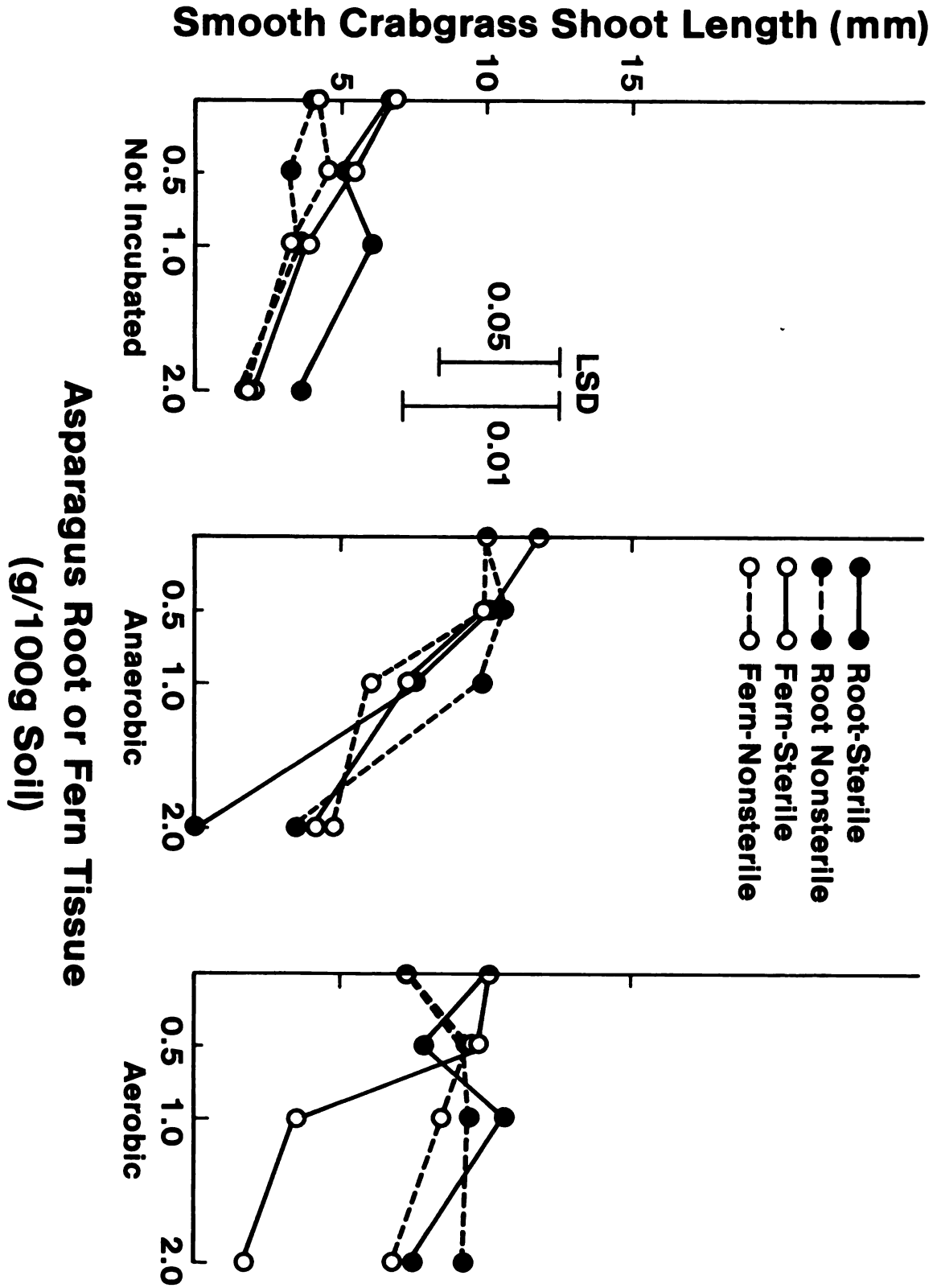
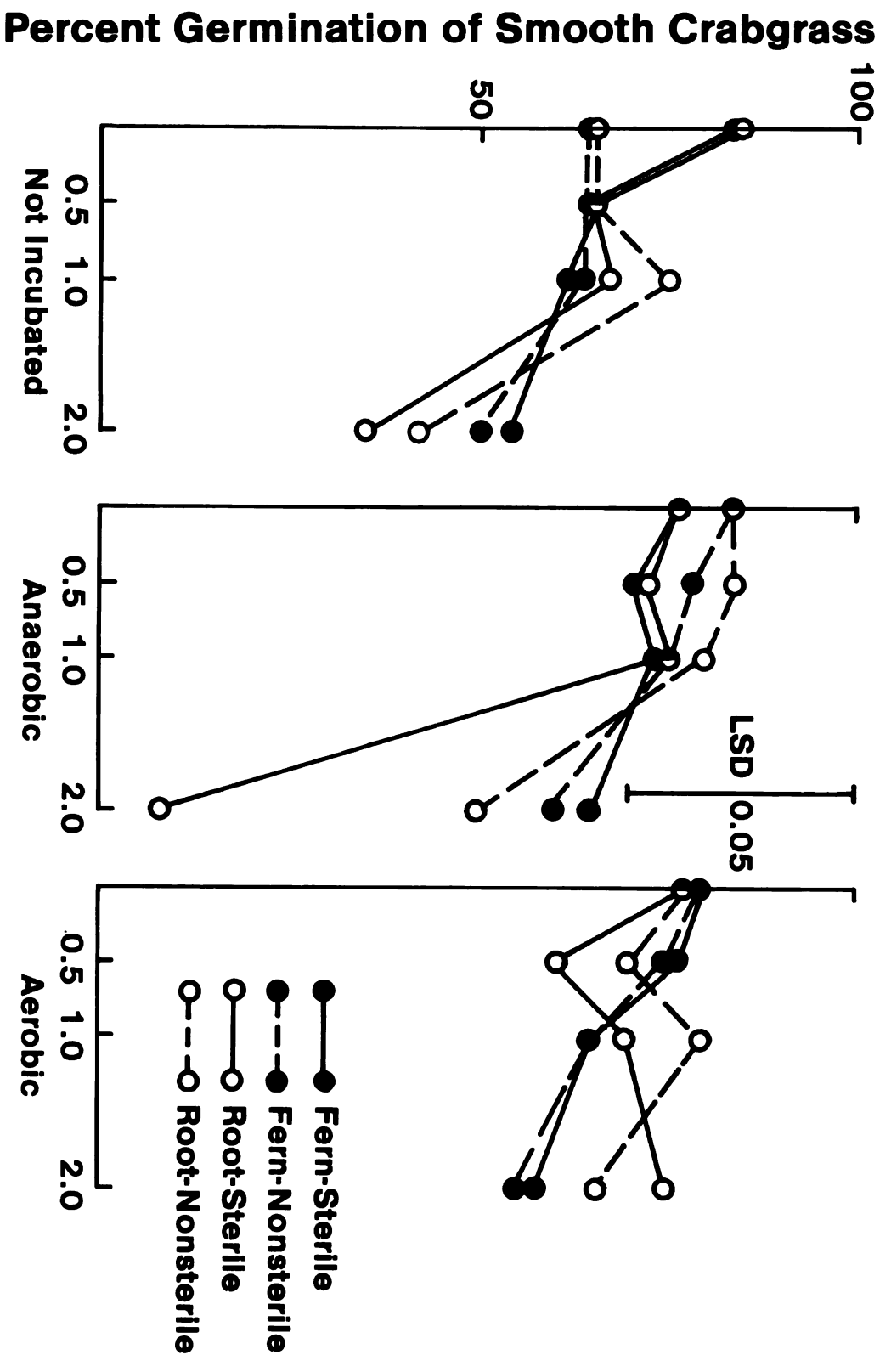

Smooth Crabgrass Shoot Length (mm)
5
10
15
0.5
1.0
2.0
Not Incubated
LSD
0.05
0.01
Anaerobic
Aerobic
Root-Sterile
Root Nonsterile
Fern-Sterile
Fern-Nonsterile
Asparagus Root or Fern Tissue
(g/100g Soil)

Figure 5: Percent germination of smooth crabgrass in response to increasing concentrations of asparagus root or fern tissue incorporated in sterile or nonsterile soil and incubated under aerobic or anaerobic conditions. Not incubated plates were controls for the initial inhibitory activity present in asparagus tissues. Interactions of incubation type x soil condition, and tissue type x level were significant at P=0.05.


Percent Germination of Smooth Crabgrass
100
50
0.5 1.0 2.0
Not Incubated
0.5 1.0 2.0
Anaerobic
LSD 0.05
0.5 1.0 2.0
Aerobic
Asparagus Root or Fern Tissue
(g/100 g Soil)
Fern-Sterile
Fern-Nonsterile
Root-Sterile
Root-Nonsterile

inhibit growth of asparagus seedlings (Yang, 1982). In these studies, fern tissue was shown to inhibit both dicot species and one monocot species differently depending on sterility of the soil. Toxic activity present in both the tissues was also shown to be decreased in some treatments when exposed to nonsterile soil conditions. These data suggest several conclusions: a) there are probably several inhibitor compounds present in the root and fern tissue that may be responsible for allelopathic properties of asparagus. b)some plant species, e.g. lettuce possesses more tolerance to the toxic principles in the tissues, c) some of the allelochemical activity may be destroyed by microbial breakdown, or d) increased activity may occur under anaerobic conditions as shown by the inhibition of lettuce root growth by fern tissue.

**Bioassays of partially purified compounds on indicator seed species.** Purified fractions from a silica gel flash column on four monocot and six dicot plant species revealed $I_{50}$ values for root length ranging from 28 ppm for lettuce and common purslane to 130 ppm for Japanese millet (Table 3). $I_{50}$ levels for shoot length ranged from 30 ppm for common purslane to 125 ppm on Japanese millet. Only barnyardgrass was not significantly inhibited by the purified extract, which agrees with the lack of sensitivity of this species in soil bioassays. These results agree with reports concerning other allelochemicals on barnyardgrass (Barnes and Putnam, 1986; Weston and Putnam, 1986). Both foxtail millet and tomato had the high $I_{50}$ levels for root length, both had significant decreases in percent germination (41% for foxtail millet, 18% for tomato. Germination of all other species was

Table 3: Inhibition of root and shoot length of indicator species in response to increasing concentrations of a purified asparagus root extract.

| Species | $I_{50}$ (ppm)[a] Root Length (mm) | Shoot Length (mm) | Germination[b] |
|---|---|---|---|
| Lactuca sativum | 28 | 37 | ns[c] |
| Raphanus sativus | 36 | 105 | ns |
| Amaranthus retroflexus | 68 | 80 | ns |
| Lepidium sativum | 37 | 90 | ns |
| Portulaca oleraceae | 28 | 30 | ns |
| Lycopersicon esculentum | 120 | 97 | * |
| Setaria italica | 130 | 125 | * |
| Digitaria sanguinalis | 35 | 55 | ns |
| D. ischaemum | 43 | 68 | 92 ** |
| Echinochloa crusgalli | ns | ns | ns |

[a] $I_{50}$ indicates concentration of asparagus root extract where 50% inhibition of root or shoot length occurs.

[c] ns=nonsignificant
*significant at P=0.05, no $I_{50}$ can be calculated at this level.
**significant at P=0.01

unaffected except for smooth crabgrass. Smooth crabgrass germination was completely inhibited at the highest concentrations while root and shoot length were inhibited by 43 ppm and 68 ppm respectively. Shoot length $I_{50}$ levels were always greater than $I_{50}$ levels for root length in all species except for tomato. However, tomato root length was only 7.5 mm in controls.

Both root and fern tissues of asparagus contain compounds potentially inhibitory to a wide range of seedling species. Compounds important in the inhibitory activity of root tissue can be isolated and concentrated by solvent extraction and column chromatography from water extracts of asparagus root tissues. No attempt was made to determine if such isolation was possible from the fern tissues. Since inhibitory activity is not rapidly decreased when root and fern tissues are subjected to microbial breakdown, compounds released into the soil as plants naturally senesce during their normal life cycle could remain in the soil solution until leached out by rain or irrigation. The data suggest that severe inhibition of seedling growth can occur in a relatively short period of time under nonsterile soil conditions, and that microorganisms are not required to produce phytotoxic activity. Also, seedling species and tissues vary in their response to asparagus toxins suggesting several chemicals, mechanisms of action or mechanisms of defense against the toxins released by asparagus tissues.

## LITERATURE CITED


1. Barnes, J. B. and A. R. Putnam. 1986. Evidence for allelopathy by residues and aqueous extracts of Rye (Secale cereale). Weed Science. 34:384-390.
2. Bell, A. A., 1974. Biochemical basis of resistance of plants to pathogens. in: Biological Control of Plant Insects and Diseases. F.G. Maxwell and F.S. Harris, eds. Pp. 403-461. Univ. Press of Mississippi, Jackson.
3. Borner, H. 1950. The role of toxic substances in the interaction of higher plants. Bot. Rev. 16:51-65.
4. Hartung, A.C. and C.T. Stephens. 1984. Effects of allelopathic substances produced by asparagus on the incidence and severity of Fusarium crown rot. J. Chem. Ecol. 1163-1174.
5. Hartung, A. C. and A. R. Putnam. 1985. Extracts of asparagus root tissue are phytotoxic. Proceedings of the Sixth International Asparagus Symposium. E.C. Lougheed, and H. Tiessen, eds. Univeristy of Guelph. Pp. 258-266.
6. Komada, H. 1975. Development of a selective medium for quantitative isolation of Fusarium oxysporum from natural soils. Rev. Plant. Plot Res. 8:114-125.
7. Laufer, G. A., and S. A. Garrison. 1977. The effect of asparagus tissue on seed germination and asparagus seedling growth. Possible allelopathic interaction. HortSci. 12:385 (Abstr.)

8. Linderman, R. G. 1970. Plant residue decomposition products and their effects on host roots and fungi pathogenic to roots. Phytopathol. 60:19-22.

9. McCalla, T. M., and F. A. Haskins. 1964. Phytotoxic substances from soil microorganisms and residues. Bacteriol. Rev. 28:181-207.

10. Molisch, H. 1937. Der Einfluss einer pflanze auf die audere allelopathie. Jena:Fisher.

11. Putnam, A. R. and W. B. Duke. 1978. Allelopathy in agroecosystems. Ann. Rev. Phytopathol. 16:431-451.

12. Rice, E. L. 1984. Allelopathy. Academic Press. N.Y. 422 pp.

13. Rice, E. L. 1979. Allelopathy-An Update. Bot. Rev. 45:15-109.

14. Shafer, W. E., and S. A. Garrison. 1980a. Effects of decomposing asparagus root tissues on lettuce, tomato, and asparagus seed emergence. HortSci. 15:406. (Abstr.)

15. Shafer, W. E., and S. A. Garrison. 1980b. Effects of asparagus root extracts on lettuce and asparagus seed germination and growth. HortSci. 15:406-407. (Abstr.)

16. Shafer, W. E., and S. A. Garrison. 1986. Allelopathic effects of soil incorporated asparagus root on lettuce, tomato, and asparagus seedling emergence. HortSci. 21(1):82-84.

17. Tiuite, J., 1969. Plant Pathological Methods. Burgess Publishing Co. Minneapolis, Minnesota.

18. Tukey, H. B., Jr. 1969. Implications of allelopathy in agricultural plant science. Bot. Rev. 35:1-16.

19. Weston, L. A. and A. R. Putnam. 1986. Inhibition of legume seedling growth by residues and extracts of Quackgrass (Agropyron repens). Weed Science. 34:366-372.

20. Yang, Hsu-Jen. 1982. Autotoxicity of Asparagus officinalis L. J. Amer. Soc. Hort. Sci. 107:860-862.

21. Young, C. C. 1984. Autointoxication in root exudates of Asparagus officinalis L. Plant and Soil. 82:247-253.

22. Young, C. C. 1986. Autointoxication of Asparagus officinalis L. in, The Science of Allelopathy. A. R. Putnam and Chung-Shih Tang, eds., John Wiley and Sons, Inc. pp. 317.

# CHAPTER III

## CHARACTERIZATION OF INHIBITORY ACTIVITY OF ASPARAGUS ROOT EXTRACTS ON ASPARAGUS SEEDLINGS


## ABSTRACT

Asparagus decline, frequently attributed to root rot of asparagus (Asparagus officinalis L.) caused by pathogenic Fusarium spp., has been correlated with the release of toxic chemicals from senescing root tissue. Greenhouse studies showed root rot was increased when asparagus seedlings were grown in the presence of increasing amounts of dried root tissue incorporated into soil with either no pathogen or in combination with F. oxysporum f. sp. asparagi or F. moniliforme. When excised asparagus roots were treated with increasing concentrations of a water extract of dried asparagus root tissues, electrolyte efflux was increased, peroxidase activity decreased linearly and respiration was decreased. Active components in the extracts were heat stable. Data suggest allelochemicals of asparagus have a direct effect on the asparagus plant and are not necessarily produced by microbial intervention.

## INTRODUCTION

Recent investigation of the causal factors of asparagus (Asparagus officinalis L.) decline has suggested that allelopathic compounds are present in asparagus root tissue (3, 4, 6, 10, 11, 12, 15, 16, 17). Several studies have indicated that allelochemicals are released from both the growing asparagus plant and from the senescing root tissue (6, 10, 12, 15, 16, 17). Toxic activity has also been shown to persist in soil and affect growth of many species including asparagus (11, 12). Also, evidence indicates that these compounds increase the incidence of asparagus root rot caused by Fusarium oxysporum (Schlecht.) Snyd. and Hans. f. sp. asparagi Cohen, and F. moniliforme Sheldon Snyd. and Hans. (3). These previous studies did not attempt to quantify the response of the asparagus plant to the level of root tissue in the soil, nor to elucidate the mode of action toxic compounds in the root tissue may have on asparagus. This study was directed at 1) quantifying the response of asparagus seedlings to root tissue incorporated in the soil with or without presence of the Fusarium pathogens, 2) developing meaningful bioassays to determine the physiological and biochemical responses of asparagus to autotoxic components from the root tissues and 3) evaluate inhibitory activity and its stability in different aged root tissues.

## MATERIALS AND METHODS

**Preparation of Asparagus Plant Material.** A commercial asparagus field (20 yr old) in Oceana County, MI was excavated and asparagus crowns (cv. Martha Washington) were collected and washed. The storage and fibrous roots were separated from the rhizomes and all dead and visably diseased plant material was discarded. The roots were oven-dried at 50 C and ground in a Wiley Mill (mesh screen size=1 mm). All plant tissues were sterilized with propylene oxide gas (14). Containers with sterilized root tissue were allowed to exhaust under a fume hood for 24 hr to dissipate any residual propylene oxide. Dried sterilized tissue was stored in brown glass bottles at -20 C until used for chemical isolation or bioassays. The tissue was checked for microbial contamination by plating 1 g of sterilized tissue from each container on Komada's Medium (5), a selective medium specific for Fusarium spp. or potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI.) for other microbial species. There were three replica plates for each container and plates were examined each day for a period of ten days for the presence of microbial colonies.

**Preparation of Fusarium Inoculum.** Millet seeds (Setaria italica (L.) (250 g) and distilled water ($DH_2O$) (100 ml) were placed in a 1 liter flask and tinsdillated (1 hr each time), then inoculated after cooling with a 4 mm diameter plug of actively growing mycelium of F.

moniliforme (FM) or F. oxysporum f. sp. asparagi (FOA). The seed inoculum was incubated at 26 C and each flask was shaken daily to facilitate mycelial spread throughout the millet seeds. After two weeks of incubation, the inoculum was air dried, then stored in paper bags at 26 C until used in soil amendment studies.

**Inhibition of Asparagus Seedling Growth by Root Tissue.** Previous research indicated when asparagus is planted in soil amended with sterilized dried root or crown tissues in the presence or absence of FOA or FM, there is a significant increase in the severity of Fusarium root rot and less growth of the asparagus plant (3). Since root tissue was shown to be the most toxic, a 4 x 3 factorial experiment was performed using 0, 5, 10, and 20 g root tissue with either no pathogen or in combination with FM or FOA. Dried root tissue was incorporated into steamed sand (Psammentic Hapludalf, sandy, mixed mesic) with or without the millet seed inoculum (8 g/pot), poured into 4 inch clay pots and three month old hybrid asparagus seedlings (cv. "UC 147") planted in the amended mix. Sterilized, uninoculated millet seed was added in the control treatment. There were 6 plants per treatment. The inoculated seedlings were placed in a completely randomized design on a greenhouse bench and watered daily. The plants were fertilized twice with soluble fertilizer (20:20:20) during the duration of the experiment. After 8 wks, the plants were harvested and evaluated visually for root rot using a scale of 1-5, where 1=no root rot, and 5=death of the plant. All ratings were assigned without knowledge of the specified treatment. The data were subjected to analysis of variance and the means separated by the LSD test.

**Biochemical Assays on Asparagus Seedlings.** Because greenhouse experiments suggest that the root tissue may be damaging the asparagus plant directly, three bioassay methods were used to further clarify the type of damage which might be incurred on the asparagus plant. Bioassays were developed using water extracts of root tissue to evaluate the effect of toxic root tissue on electrolyte efflux, respiration and peroxidase activity. These methods were chosen to evaluate if asparagus tissue damaged through cellular disruption or plant energy processes. Preliminary experiments for each assay were performed to determine optimum concentrations of root extract necessary for each test. In electrolyte efflux studies, asparagus storage and fibrous root tissue from 1 month-old asparagus seedlings (cv. UC 147), was cut into 2 mm sections, weighed into 200 mg fresh weight samples and placed into 20 ml vials. Water extract of dried asparagus root tissue (2.0 and 5.0 mg/ml) was added to the vial. The root extract had been previously filter sterilized (Nalgene 0.2 m disposable filterware) and a deionized $DH_2O$ control was also included in the experiment. The vials were vacuum infiltrated by reducing the air pressure to about 2 cm Hg for 15 min, then the extracts were decanted from the tissue, and the samples were rinsed three times in deionized $DH_2O$. Deionized $DH_2O$ (10 ml) was then added to the vials and samples placed on a reciprocal shaker (100 strokes/min). Conductance of the ambient solutions were determined at intervals with a conductivity meter equipped with a pipet-type electrode assembly. The conductance value for root tissue in the deionized $DH_2O$ controls was used as a correction factor to calculate root extract induced electrolyte efflux. There were 3 replications per treatment and the experiment was repeated

2 times. Data presented are the results from one representative experiment.

In respiration studies, fresh asparagus root tissue was removed from one year-old crowns (cv. Viking) and cut into 1 cm segments, separated into 1 g samples, and immersed in a 300 µg/ml solution of either a sterilized water extract of asparagus root tissue or $DH_2O$. The tissue was infiltrated for 15 min under vacuum pressure as above. Entire roots were also cut from the asparagus rhizome, separated into 1 g samples, and either submerged for 1 hr in asparagus root extract (300 µg/ml ) or $DH_2O$ in 20 ml vials or left intact in the vials and not submerged. All solutions were decanted from the tissues. Oxygen uptake was monitored using a Gilson Respirometer (20 C) at 20 min intervals over a period of 5 hr. There were 3 replications for each treatment, and the experiment was repeated 2 times. Data presented are results from one representative experiment. The data were subjected to an Analysis of Variance and the means separated by Duncans's Multiple Range Test.

One month-old seedlings (cv. UC 147) were infiltrated with 0, 10, 100 or 1000 µg/ml solution of asparagus root extract and monitored over time for peroxidase activity. Peroxidase was assayed by the method of Ridge and Osborne (8) and expressed as the change in $A_{470}$/min-1 g dry weight of asparagus root tissue. There were 3 replications for each treatment. The data were subjected to an Analysis of Variance and the means separated by the LSD test.

**Comparison of Inhibitory Activity from Different Aged Asparagus Plants.** Asparagus crowns (cv. Martha Washington) were excavated from plantations 5, 12, and 20 yrs old and prepared as previously described.

Dried root tissue (25 g) from each field was extracted with 500 ml $DH_2O$ overnight at 4 C. The particulates were removed by straining each sample through 3 layers of cheesecloth and centrifuging for 20 min at 6,000 g. The samples were then decanted from the pellet and lyophilized to determine the dry weight, resolvated in $DH_2O$ at known concentrations (0.0, 0.5, 1.0, 2.0 mg/ml) and applied to petri dishes (60 x 15 cm). Previous studies in this laboratory have shown that Lepidium sativum L. (cv. Curly Cress) is a fast and reliable seedling for evaluating the inhibitory activity of allelopathic compounds (7). Therefore, curly cress was used to test inhibition from root extracts of asparagus tissues of increasing ages. Curly cress seeds (10/dish) were added to the dishes along with 1.5 ml $DH_2O$, then incubated for 72 hr at 26 C in a growth chamber. There were 3 replications per treatment and the treatments were arranged in a randomized complete block design. Root and shoot lengths were measured and $I_{50}$ levels determined by interpolation. The data were subjected to an Analysis of Variance and the means separated by the LSD test.

Part of each sample was used to determine if the compounds present in the extract were heat stabile. Each sample (50 ml) was autoclaved 20 min at 120 C at 23 psi then applied to petri plates (60 x 15 cm) at known concentrations (0.0, 0.5, 1.0, 2.0 mg/ml) and bioassayed using curly cress as above. Unautoclaved samples at equal concentrations and controls were also bioassayed as indicated above.

## RESULTS

**Preparation of Asparagus Plant Material.** In allelopathy studies, it is important to separate the contribution of chemical compounds produced by plant tissues as they are degraded by resident microbes and compounds released directly from plant tissues. Therefore it was important to sterilize plant tissues used in all experiments to remove the confounding factor of microbes. After asparagus root and fern tissues were treated with propylene oxide gas, then plated out on either Komada's medium or PDA, no Fusarial or other microbial colonies were detected.

**Inhibition of Asparagus Seedling Growth by Root Tissues.** When root tissue was incorporated into the soil, increasing levels of tissue resulted in significantly increased levels of root rot on asparagus seedlings (Figure 1). When either of the Fusarium pathogens were incorporated in combination with the root tissue, there was a significant increase in the level of root rot on the asparagus plant. Analysis of variance of these data indicated significant main effects from the root tissue and the pathogens on the asparagus plant.

**Physiological and Biochemical Assays on Asparagus Seedlings.** Electrolyte efflux increased at the very highest (5 mg/ml) crude extract levels ($P=0.01$) as well as the 2mg/ml level $P=0.05$ (Figure 2).

Figure 1: Root rot ratings of asparagus seedlings (cv. UC 147) grown in soil amended with increasing levels of dried asparagus root tissue alone or in combination with F. oxysporum f. sp. asparagi or F. moniliforme. Control was soil amended with sterilized millet seed only or increasing concentrations of dried asparagus root tissue.

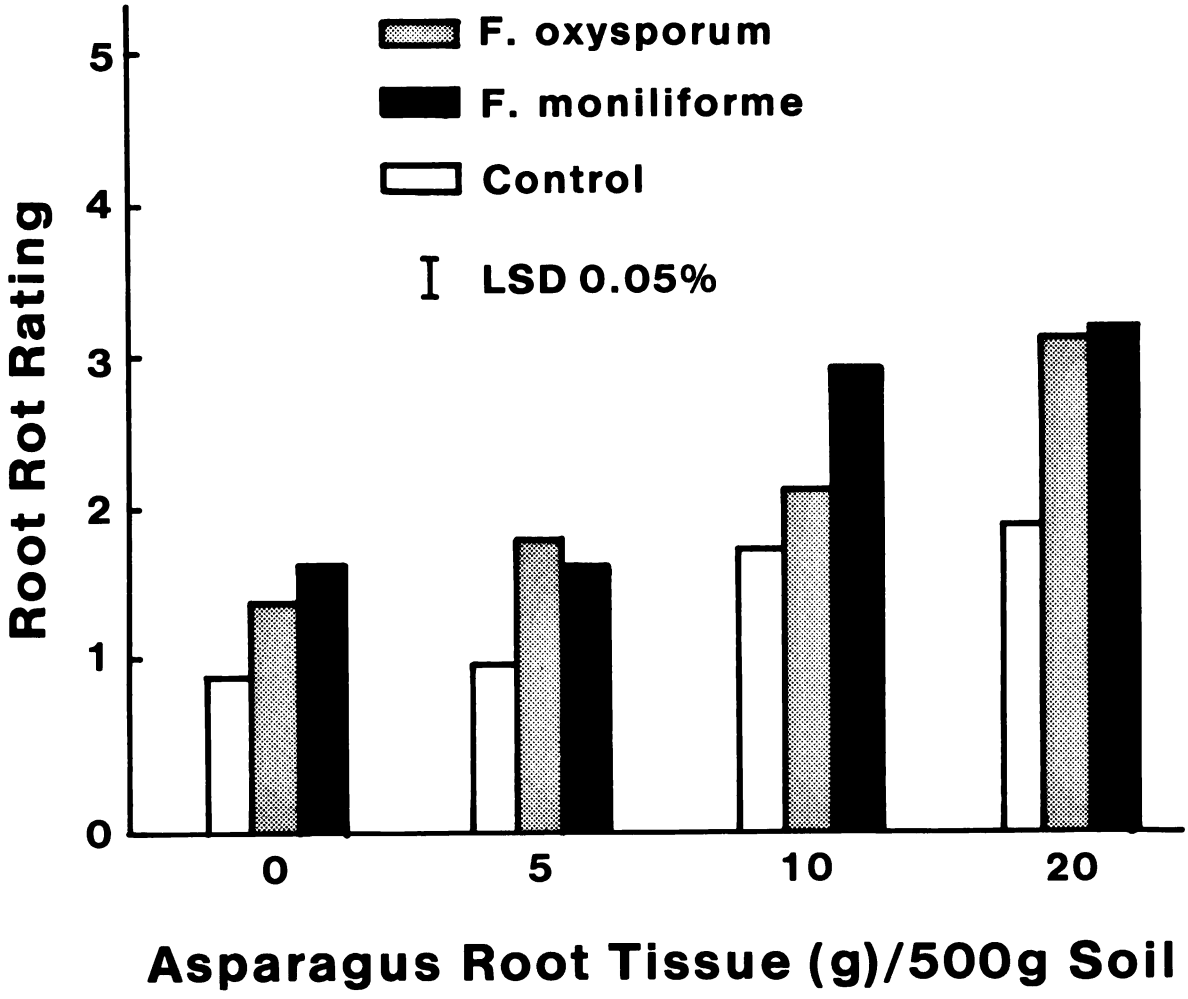
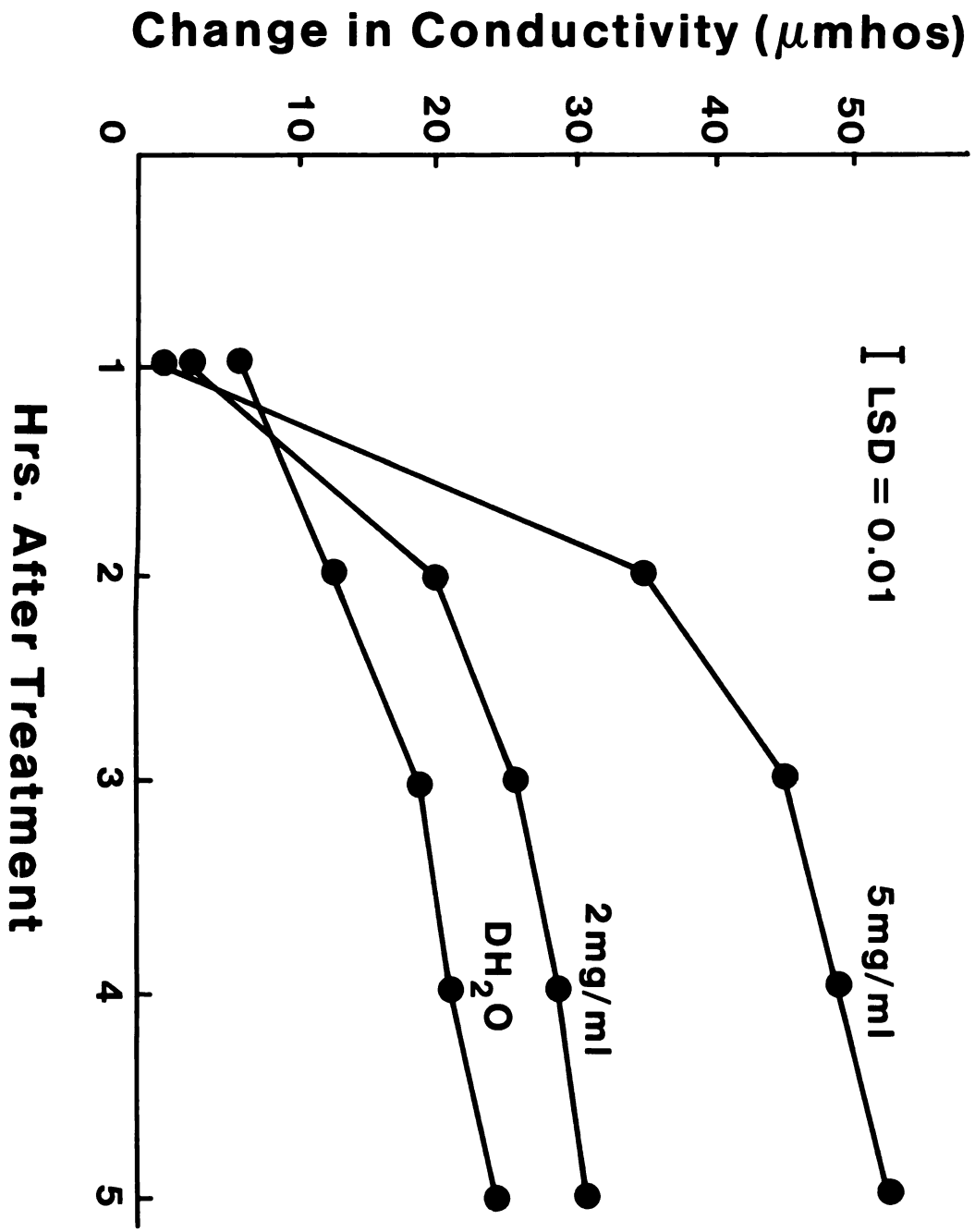

F. oxysporum
F. moniliforme
Control
LSD 0.05%
Root Rot Rating
0
1
2
3
4
5
0
5
10
20
Asparagus Root Tissue (g)/500g Soil

Figure 2: Electrolyte efflux over time from excised asparagus root tissue treated with increasing concentrations of a water extract of asparagus root tissue.


Change in Conductivity (μmhos)
0
10
20
30
40
50
1
2
3
4
5
Hrs. After Treatment
LSD = 0.01
5mg/ml
2mg/ml
DH2O

Electrolyte efflux was also tested at concentrations similar to those used in other biochemical assays (data not shown) but no significant increase in leakage was observed. No attempt was made to determine ion influx or exchange.

Peroxidase activity was decreased over time when the seedlings were subjected to increasing concentrations of the crude root extract (Figure 3).

In respiration experiments, the control tissues ( not submerged in $DH_2O$) exhibited higher rates of $O_2$ uptake than other treatments throughout the experiment (Table 1). In tissue subjected to the aqueous root extracts, respiration was significantly less than in the $DH_2O$ treatments. Infiltration alone significantly reduced respiration as compared to the control tissue. However, infiltration with the aqueous root extracts reduced respiration considerably more than infiltration with $DH_2O$. Not suprisingly, submerging the root tissue in distilled water also reduced its respiration, but not differently than when the tissue was infiltrated with $DH_2O$.

**Comparison of Inhibitory Activity From Different Aged Asparagus Plants.** Both root and shoot lengths of cress seed were inhibited by increasing levels of asparagus root extracts, but root length was consistantly the more sensitive parameter ( average $I_{50}$ for root length=1083 µg/ml, average $I_{50}$ for shoot length=1644 µg/ml) (Figure 4). Both root and shoot bioassays indicated that the tissue from the 5 yr-old plants contained the most inhibitory activity, whereas the 12 yr-old and 20 yr-old were less active but not significantly different from each other (LSD=3.60 at 0.01 level for both root and shoot length). Root length was inhibited the most by the 5 yr extracts over all dilutions but was

Figure 3: Peroxidase activity of asparagus seedlings infiltrated with increasing concentrations of a water extract of asparagus root tissue.

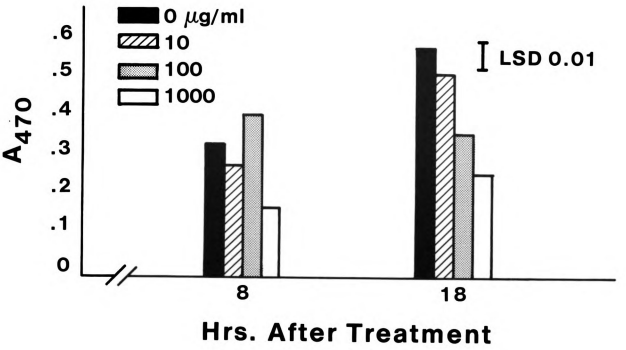
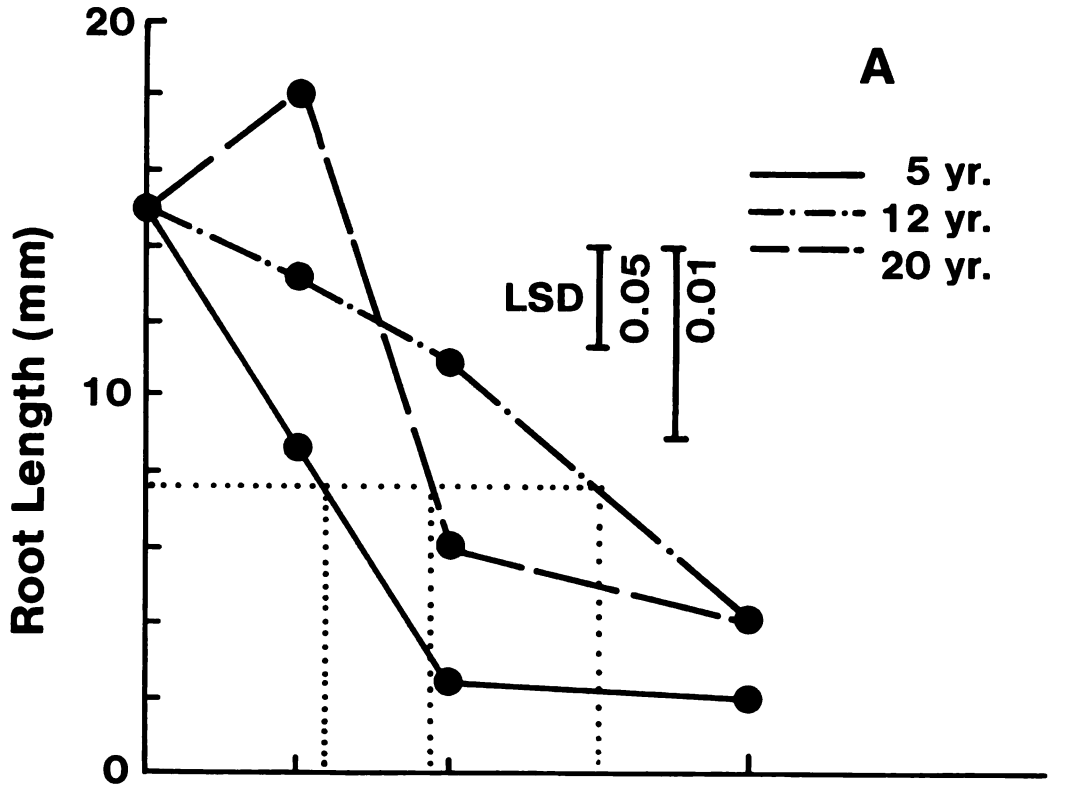

0 µg/ml
10
100
1000
LSD 0.01
A470
.6
.5
.4
.3
.2
.1
0
8
18
Hrs. After Treatment

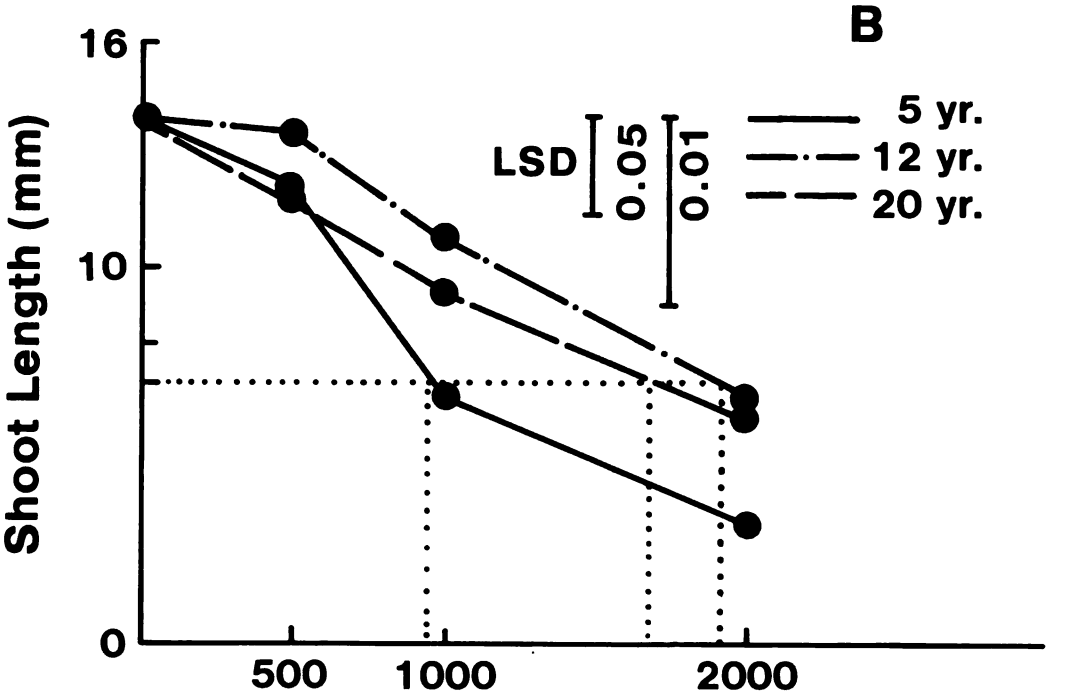
Table 1: $O_2$ uptake after infiltration of asparagus root tissues with distilled water or asparagus aqueous extracts (300 µg/ml), or from submerging excised asparagus roots in aqueous extracts or distilled water after five hr.

| Treatment | $O_2$ Uptake (µl/g-hr)[z] |
|---|---|
| Infiltrated with root extract | 46.2 a |
| Infiltrated with distilled water | 64.6 b |
| Submerged in root extract | 39.4 a |
| Submerged in distilled water | 61.1 b |
| Not submerged | 112.0 c |

[z]Means with uncommon letters differ at P= 0.05 by Duncan's Multiple Range Test.

Figure 4: Root and shoot length of curly cress seedlings treated with increasing concentrations of a water extract of asparagus root tissue from 5, 12, and 20 yr old plantations. A=Root Length, B=Shoot length. Dotted line=$I_{50}$ levels.




**Asparagus Root Extract ($\mu$g/ml Dry Weight)**

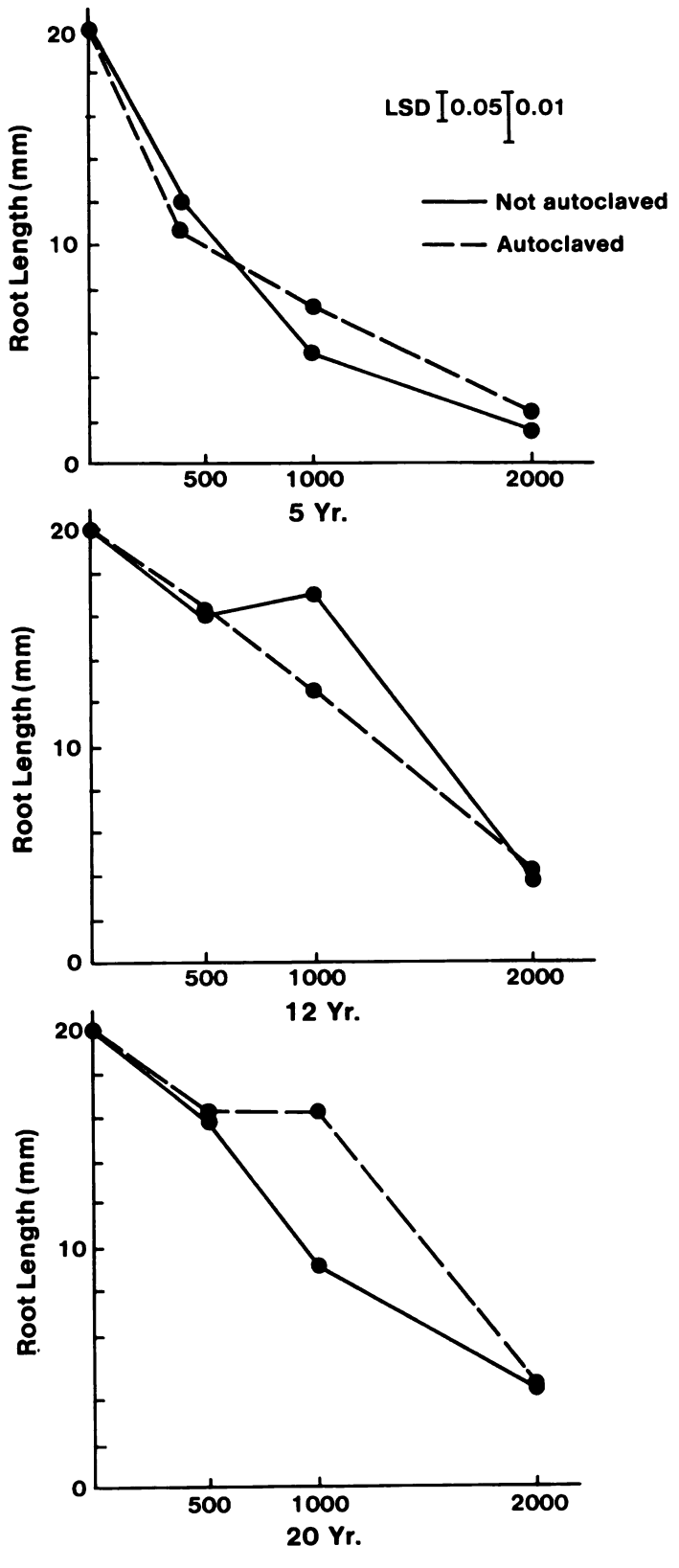
not significantly different from the 12 and 20 yr-old treatments at the highest concentration. Measurements of shoot length showed that activity from the 12 and 20 yr extracts was not significantly different from each other ($I_{50}$=1950 µg/ml in 12 yr and 2000 µg/ml in 20 yr treatments). The 5 yr-old tissue extracts had the most inhibitory activity on shoot growth ($I_{50}$=983 µg/ml).

Autoclaving extracts from the 5 and the 12 yr-old tissue tissue had no significant effect on inhibitory activity (Figure 5). Autoclaved extracts from the 20 yr-old tissue showed less activity than the non-autoclaved extracts at the intermediate level but did not differ at the highest or lowest levels.

## DISCUSSION

Root rot on asparagus is significantly increased when asparagus seedlings are grown in the presence of increasing levels of dried asparagus root tissue with or without FOA or FM. Dried fern tissue was also tested in a similar fashion in prior experiments did not provide the same increase in disease as was noted with the root tissue (3). These data suggest that a chemical or physical property of the root tissue influences the incidence of Fusarium root rot on the asparagus seedlings. The data also suggests that this is a direct effect of the residues on the asparagus plants and not an interaction of the residues and the pathogens. In experiments with sterilized and nonsterilized vermiculite, Young (18) found that asparagus seedlings planted in unsterilized "used" vermiculite and treatments with root tissues alone started to "yellow and wilt" after 21 days but were normal in controls

Figure 5: Root length of curly cress treated with increasing concentrations of autoclaved and nonautoclaved water extract of asparagus root tissues from 5, 12, or 20 yr old plantings.


20
10
0
Root Length (mm)
LSD 0.05 0.01
Not autoclaved
Autoclaved
500
1000
2000
5 Yr.
12 Yr.
20 Yr.
Asparagus Root Extract (μg/ml Dry Wt)

and treatments with sterilized vermiculite and root tissues. He postulated that there is an interaction between the residues and microorganisms. However, the exact nature of the "used vermiculite" or its microbial content was not published, making it difficult to assess the results. Our data support the hypothesis that root rot on the crown is increased due to damage to the root system that could be caused by toxic components released from senescing root tissue rather than from microbial intervention.

Although asparagus has been reported to release autotoxic compounds from the root tissues as well as from intact root systems, no data has been presented concerning the nature of the autoxicity or the site of action. Three types of assays were used to investigate what processes could be affected by root extracts; electrolyte efflux to test for tissue perterbation (1), peroxidase activity, as an increase in peroxidase as been associated with increased disease resistance (2), and respiratory activity of young asparagus root tissue. In electrolyte efflux studies, efflux occurred only at much higher extract concentrations than where responses occurred in the other bioassays. These results suggest that compounds important in tissue perturbations are at low concentrations, that asparagus tissues are not sensitive to the compounds in the crude extract or other compounds in the complex mixture of the crude extract could be confounding the experiment. Peroxidase activity was significantly decreased after 18 hr. Since an increase in peroxidase is associated with disease resistance, these data may suggest that the plant's defense mechanisms to combat infection from its pathogens are reduced in the presence of root tissue, making the plant more susceptible to disease. Respiration was

also significantly reduced in one year-old root tissues indicating that compounds present in the extracts are damaging the energy-producing processes of young roots. These data all support the hypothesis that chemicals present in the water extracts are altering the plant's biochemical processes.

In experiments to test different aged tissues, it may not be surprising that tissue from 12 and 20 year-old plants did not vary in toxicity since the storage root tissues of the asparagus crown is replaced approximately every 6 years (9, 13). Although plants were removed from fields of increasing age, tissue present in these fields may vary in age (9, 13). Although tissue collected from younger plantings was more toxic than tissue collected from older plantings, it is difficult to deduce from these experiments if tissue age was the determining factor. Chemicals may be detoxified or leached from the tissue as tissues age, but other expermentation is needed to assess the true effects of tissue age on tissue toxicity. Compounds present in tissues are heat stable, suggesting that the components may be difficult to destroy in a natural agroecosystem. Shafer and Garrison presented data indicating that toxicity is present in the soil for up to 90 days (12). With their data, it is difficult to determine if this loss of toxicity is due to microbial breakdown or leaching of the compounds from the rhizosphere. Data collected in this laboratory suggests that the compounds important in the autotoxic responses have a pronounced polar nature and therefore may be solvated from the soil system by leaching (3, 4). We have reported previously that root tissue did not affect the growth of FOA or FM but will affect other microorganisms (3). Therefore replant problems in old asparagus plantations may be attributed to the continual presence of Fusarium

pathogens once the crowns have been destroyed rather than long term persistence of toxins in the soil.

## LITERATURE CITED


1. Bronson, C. R. and R. P. Scheffer. 1977. Heat- and aging-induced tolerance of sorghum and oat tissues to host-selective toxins. Phytopath. 67:1232-1238.
2. Hammerschmidt, R., E. M. Nuckles, and J. Kuc. 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to Colletotrichum langenarium. Physiol. Plant Phys. 20:73-82.
3. Hartung, A. C., and C. T. Stephens. 1983. Effects of allelopathic substances produced by asparagus on the incidence and severity of Fusarium crown rot. J. Chem. Ecol. 9:1163-1174.
4. Hartung, A. C. and A. R. Putnam. 1985. Extracts of asparagus root tissue are phytotoxic. Proceedings of the Sixth International Asparagus Symposium. E. C. Lougheed, and H. Tiessen, eds. University of Guelph. p. 258-266.
5. Komada, H. 1975. Development of a selective medium for quantitative isolation of Fusarium oxysporum from natural soils. Rev. Plant. Plot Res. 8:114-125.
6. Laufer, G. A., and S. A. Garrison. 1977. The effect of asparagus tissue on seed germination and asparagus seedling growth. Possible allelopathic interaction. HortSci. 12:385 (Abstr.)

7. Lehle, F. R. and A. R. Putnam. 1982. Quantification of allelopathic potential of sorghum residues by novel indexing of Richards' Function fitted to cumulative cress seed germination curves. Plant Physiol. 69:1212-1216.

8. Ridge, A. and S. Osborne. 1970. Hydroxyproline and peroxidase activity in cell walls of Pisum sativum: Regulation by ethylene. J. Exp. Bot. 21:843-856.

9. Robb, A. R. 1984. Physiology of asparagus (Asparagus officinalis) as related to the production of the crop. New Zealand J. Exp. Agri. 12:251-260.

10. Shafer, W. E. and S. A. Garrison. 1980. Effects of decomposing asparagus root tissues on lettuce, tomato, and asparagus seed emergence. HortSci. 15:406. (Abst.)

11. Shafer, W. E. and S. A. Garrison. 1980. Effects of asparagus root extracts on lettuce and asparagus seed germination and growth. HortSci. 15:406-407. (Abstr.)

12. Shafer, W. E. and S. A. Garrison. 1986. Allelopathic effects of soil incorporated asparagus roots on lettuce, tomato, and asparagus seedling emergence. HortSci. 21(1):82-84.

13. Tiedjens, V. A. 1926. Some observations on root and crown bud formation in Asparagus officinalis. Proc. Am. Soc. Hort. Sci. 23:189-195.

14. Tuite, J. 1969. Plant Pathological Methods. Burgess Publishing Co. Minneapolis, Minnesota.

15. Yang, Hsu-Jen. 1982. Autotoxicity of Asparagus officinalis L. J. Amer. Soc. Hort. Sci. 107:860-862.

16. Yang, Hsu-Jen. 1985. Autotoxic and allelopathic characteristics of Asparagus officinalis L. Proceedings of the Sixth International Asparagus Symposium. E. C. Lougheed and H. Tiessen., eds. University of Guelph. p.267-276.

17. Young, C. C. 1984. Autotoxication in root exudates of Asparagus officinalis L. Plant and Soil. 82:247-253.

18. Young. C. C. 1986. Autotoxication of Asparagus officinalis L. In: The Science of Allelopathy. A.R. Putnam and Chung-Shih Tang ed. John Wiley and Sons, Inc. 317 pp.

# Chapter IV

# IMPACT OF ASPARAGUS AND ASPARAGUS PLANT TISSUES ON SOIL MICROBE POPULATIONS

## ABSTRACT

The responses of microbial populations in soils with dried asparagus root or fern tissue or soils planted with asparagus (Asparagus officinalis L. cv UC 147), A. sprengeri, snap beans (Phaseolus vulgaris cv. Bush Blue Lake) or sweet corn (Zea mays cv. Calico King) were evaluated. Fusarium spp. and other fungal populations increased in treatments where asparagus root tissue was incorporated into soil. In contrast, bacterial species quickly colonized fern tissue. Both bacterial and fungal populations were significantly lower in treatments where asparagus plants were grown but not in treatments where snap beans or sweet corn were planted. Nine isolates of Pythium spp., and 6 of 18 bacterial isolates were inhibited by purified components from asparagus root tissue.

## INTRODUCTION

Plant residues from various sources contribute to the organic matter component of soils. These tissues from young, mature, senescing and dead plant parts are ultimately decomposed in the soil by the

resident microbial populations or abiotic factors. Thus, chemical components bound in the plant materials are made available for microbial and plant uptake. At any one time, the soil may contain a vast array of chemical components which may affect plant growth in either beneficial or detrimental ways.

Asparagus (Asparagus officinalis L.), an important vegetable crop in Michigan, is often plagued with two major problems; a) crown death from a root rotting disease caused by F. oxysporum f. sp. asparagi (FOA), and F. moniliforme (FM), (1, 2) and b) decline and unprofitable yields from replanted fields. (4, 5, 23). The two problems have been collectively termed "Asparagus Decline". Recent research suggests that, although decline is ultimately caused by pathogenic Fusarium species, asparagus also contains compounds that are autotoxic or autoallelopathic, and these compounds may be attributing to the severity of the decline problem (6, 7, 9, 20, 21, 22, 25, 26, 27). Allelopathy is defined as a direct or indirect harmful affect by one plant (including microorganisms) on another through the production of chemical compounds released into the environment (18).

Decomposition of plant products in the soil associated with the formation of phytotoxic substances has been documented by numerous reports (3, 11, 12, 14, 15, 23, 24). One mechanism by which allelopathic compounds may indirectly affect plant growth is by affecting the microbial populations in the soil leading to an ecological imbalance of the naturally occuring microflora. This imbalance can result in an increased level of disease on crop plants. Tousson and Patrick (23) reported that the incidence of root rot of bean (Phaseolus vulgaris) caused by F. solani (Mart.) was increased if the roots were exposed to extracts from decomposing residues of rye,

barley, broccoli, and broad bean. Patrick and Koch (16) reported that exposure of tobacco to leachates from decomposing rye and timothy residues increased the susceptibility of both resistant and susceptible varieties of tobacco to black root rot caused by Thielaviopsis basicola (Beck. and Br.) Ferraris.

Preliminary observations have indicated that microbial populations in asparagus fields are reduced when compared to microbial populations in similar soils planted with other crops (Putnam et. al., unpublished data). Also, we have reported previously that extracts of asparagus root tissues are inhibitory to Pythium spp. but not to either FOA or FM (6). Based on these data, it was hypothesized that toxic substances released from senescing asparagus tissues may be harmful to some organisms but not to the pathogenic species of Fusarium, thus creating a more favorable environment for pathogenesis to occur. The purpose of this research was a) to determine how asparagus root or fern residues incorporated in the soil would affect natural microbial populations, b) to determine if the presence of asparagus plants as compared to other plants (e.g. snap beans or corn) could affect populations and diversity of microbial species in the soil and c) to directly test phytotoxic extracts of asparagus root tissue for inhibitory activity on isolated bacterial or fungal species.

## MATERIALS AND METHODS

**Impacts of various plant tissues on naturally occuring microbial populations.** Asparagus root and fern tissues collected, ground, and sterilized as previously reported (6) were incorporated (50g/180 g

soil) into Spinks sandy loam soil (Psammentic Hapludalf, sandy, mixed, mesic, with 1% organic matter, pH 6.5) and the soil placed in 15.2 cm clay pots. In a separate treatment, shredded poplar excelsior (50 g) was also incorporated into soil as a control for the physical influence of organic matter in the soil. Another treatment used a living asparagus crown ( UC 157, 1 yr old) planted a similar pot. There were 2 plants per treatment and 3 replications of each treatment. The pots were placed on a greenhouse bench and subirrigated as necessary to maintain equal moisture levels in all treatments. Unwanted plant growth was suppressed by hand weeding. Plants were fertilized once a week with soluble fertilizer (20:20:20) during the duration of the experiment. Microbial populations were monitored at 1, 4, 8, 12, 18, and 22 weeks.

In a second experiment, treatments were the same except snap beans (Phaseolus vulgaris L. cv. Bush Blue Lake) and sweet corn (Zea mays cv. Calico King) were included as plant controls and the poplar excelsior treatment was omitted. Samples were taken at 2, 5, 9, 18, and 22 weeks.

To monitor changes in the microbial populations in the soil over the duration of the experiment, the soil was first analysed for total microbial population before the experiment was initiated. The baseline populations of microbial organisms were determined from three random samples from the soil. Data for total bacterial (sensu stricto), fungi, actinomycetes, and Fusarium spp. populations were all monitored. A dilution series was made in normal saline and samples plated on selective media. To evaluate changes microbial populations over time, soil samples were taken from each pot by removing the top 2 cm of soil, then taking 4 samples from each pot with an 8 mm diameter cork borer.

The 4 cork borer samples were mixed well, allowed to air dry, then sieved through a 40 mesh sieve (425 u) to remove any plant material, and weighed into 1 g samples. Each sample was mixed with 9 ml of sterile normal saline (0.85% aqueous NaCl) and vortex-mixed for 30 sec. The particulates were allowed to settle and serial 10-fold dilutions were made. Aliquots of 0.1 ml from each dilution were plated on media selective for bacteria, fungi, actinomycetes, and Fusarium spp. The Petri plates were incubated at 25 C for 3 days for enumeration of bacteria, 4 days for fungi, 7 days for actinomycetes, and 10 days for Fusarium, then discrete colonies were counted. There were 2 dilutions per sample and 2 replica plates for each dilution. Microbial counts from the 2 replica plates were averaged to obtain the number of colony forming units (CFU) per gram of soil. Data were subjected to an Analysis of Variance for each time period and the means separated by LSD test.

**Selective media used for evaluation of microbial species in greenhouse experiments.** The medium used for bacteria was nutrient agar (Difco Laboratories, Detroit, MI); 2 ml nystatin, (15 mg/ml in DMSO), and 28 ml cycloheximide, (15 mg/ml in Distilled $H_2O$ ($DH_2O$)). Solutions were added to one liter of medium after autoclaving. Fungal populations were evaluated on Czapeks' agar (30 g sucrose, 2 g sodium nitrate, 1 g dipotassium phosphate, 0.5 g magnesium sulphate, 0.5 g potassium chloride, and 0.01 g ferrous sulfate in 1 L $DH_2O$ amended with rose bengal (35 mg/L to restrict colonies) and chloramphenicol (100 mg/L to suppress bacteria). Populations of actinomycetes were evaluated on a selective medium containing 3 g N.Z.-amine-A (Schefield Chemicals,

Norwick, NY) , 18 g bacto agar (Difco Laboratories, Detroit, MI), in 1 L tap $H_2O$; 5 ml bromocreosol green (0.04% in $DH_2O$) was added to restrict the colonies after adjusting the pH to 7.2. Antifungal antibiotics nystatin and cycloheximide were added asceptically as described above. For estimating populations of Fusarium spp. Komada's medium was used (8). The medium contained potassium monophosphate, 1.0 g, magnesium sulphate + 7 $H_2O$, 0.5 g, potassium chloride, 0.5 g, Fe-Na EDTA, 0.01 g, L-asparagine, 2.0 g, D-galactose, 20 g, sodium borate + 10 $H_2O$, 1.0 g, oxgall, 0.5 g, PCNB (75%)(Terachlor), 1.0 g, Bacto agar, 15 g, added to 1 L of $DH_2O$ and steamed for 1 hr. The pH was adjusted between 3.8-4.0 with 10% phosphoric acid. Chloramphenicol, (0.25 g/L), was added after the medium had cooled.

**Preparation of partially purified asparagus root extracts.** Since other laboratories as well as this one have published that water extracts of root tissue are toxic to seedling germination and radical elongation, water extracts from root tissue were subjected to solvent extraction and thin layer chromatography (TLC) to try and isolate chemical components that may also be inhibitory to microbial organisms (6, 25). Ground asparagus root tissue was extracted by stirring overnight at 4 C (1:10 root tissue:$DH_2O$). The particulates were then removed by filtering through four layers of cheese cloth and centrifuging at 6,000 x g for 20 min. The supernatant was decanted carefully from the pellet (the pellet was discarded) and then precipitated with acetone (4:1 v:v acetone:sample) overnight at 4 C. The precipitant was discarded and the liquid concentrated to 1/4 the original volumn on a Buchi Rotary evaporator. This concentrate was extracted with chloroform 3 times (1:1 v:v). The chloroform fraction

was reduced to dryness, weighed, redissolved in methanol, and filter sterilized. This extract was further purified on TLC plates (Whatman silica gel 60, 200 μ thickness, mobile phase, hexane:ethyl acetate 1:1 v:v). TLC plates were washed twice in this solvent system and allowed to dry each time before using in purification procedures. After the chloroform fraction was developed on the plates, the edge of each plate was removed and developed using 5% vanillin in concentrated $H_2SO_4$. Rf values were determined for separate spots and the remainder of the plate was scraped accordingly. Individual spots were eluted from the silica gel with 100 ml of methanol or chloroform. Samples were filtered, dried under nitrogen, then weighed and kept at -20 C until used in bioassays. A duplicate plate with no sample was also developed in the solvent system, scraped and eluted with methanol for use as a control.

**Effects of water extracts on isolates of Pythium spp.** Previously published data have indicated that asparagus root tissues are inhibitory to Pythium spp. but not to Rhizoctonia spp. or either of the Fusarium root rot organisms that attack asparagus plants (7). Pythium spp. is a well known soil pathogen and different isolates were used to test for sensitivity to water extracts of asparagus root tissue. Thirteen isolates of Pythium spp. were tested for sensitivity to water extracts of dried asparagus root tissue. Eleven isolates of P. ultimum Trow, were collected in Ohio, and one isolate each of P. ultimum and P. aphanadermatum (Edson) Fitzs., were collected in Michigan. A water extract of dried asparagus root tissue was sterilized with a millipore filter (0.22 μm) to remove any bacterial or fungal contamination. The

extract was applied to potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) plates (9 cm diameter) (0.2 ml) and allowed to dry. Each plate was then inoculated with a 2 mm agar plug of an actively growing Pythium isolate. Cultures were allowed to grow for 4 days at 25 C then visually assessed for hyphal inhibition or stimulation. There were 2 replica plates/isolate and $DH_2O$ was added to the control plates.

**Sensitivity of bacterial isolates to purified extracts of asparagus root tissue.** Naturally occuring soil bacteria were isolated from Spinks sandy loam soil using Martin's selective medium for bacteria (13). The medium contained 0.3% trypticase soybroth (BBL Microbiology Systems, Cockeysville, MD), 50 ppm PCNB, and 15 g bacto agar (Difco Laboratories, Detroit, MI) in 1 L $DH_2O$, autoclaved for 20 min then poured into 9 cm petri dishes. Individual colonies were removed from the selective medium and streaked on an amended nutrient agar. Single colonies were subcultured on agar slants and stored at 4 C until used in bacteria sensitivity tests. The amended nutrient agar medium contained 1.4 g nutrient broth (Difco Laboratories, Detroit, MI) 5 g glucose, 5 g yeast extract (Difco Laboratories, Detroit, MI), and 15 g bacto agar in 1 L $DH_2O$. All bacterial studies were done using this medium. Soft agar overlays made from this medium contained 0.15% bacto agar.

The bacterial suspension in the soft agar overlay was made by adding 1 ml of sterile normal saline to a 24 hr-old culture in a slant, vortexing 30 sec to suspend the bacteria in the saline, then using 100 µl of this suspension to inoculate the soft agar overlay.

Individual bacterial isolates were tested for sensitivity to components eluted from TLC plates. Dried components from TLC plates

were redissolved in methanol at 1.0 and 0.5 mg/ml, then 10 ul of each dilution spotted on amended nutrient agar along with a methanol and a silica gel plate control. After the methanol had evaporated from the plate, a soft agar overlay inoculated with a bacterial isolate was poured over the surface of the plate. Plates were incubated at 25 C for 48 hrs then examined for zones of inhibition in the bacterial lawn. There were 18 isolates of bacteria tested for sensitivity to isolated compounds from asparagus root tissue. All cultures that were sensitive to the extracts were identified by the Animal Health Diagnostic Laboratory, P.O. Box 30076, Lansing, MI.

## RESULTS

**Impacts of various plant tissues on naturally occuring microbial populations.** In all microbial species tested, poplar excelsior had little effect on microbial populations, indicating that the physical presence of non-nutritive tissues did not affect population levels (data not shown). Therefore this treatment was deleted from subsequent experiments.

There were no differences in bacterial populations between pots containing a living asparagus plant and the control soil in experiment one (Table 1). In pots containing root tissue, there were significant increases in the bacterial populations at 4 and 8 weeks, then populations returned to levels not significantly different from the control soil. However, there were highly significant increases in the bacterial populations in soils amended with fern tissue. Bacterial populations were significantly higher for soil amended with fern tissue

Table 1: Estimates of bacterial populations (CFU's) in Spinks sandy loam soil amended with 50 g of dried asparagus root or fern tissue or planted with asparagus, snap beans, or sweet corn. Initial bacterial population in 1 g of unamended soil was 36.8 x $10^6$ CFU in Experiment #1 and 37.4 x $10^6$ CFU in Experiment #2.

| EXPERIMENT #1 | x$10^6$ CFU/g Soil at the Time of Evaluation (weeks) | | | | | |
|---|---|---|---|---|---|---|
| Treatment | 1 | 4 | 8 | 12 | 18 | 22 |
| Control | 47.3 bc* | 87.0 c | 26.5 c | 24.5 b | 41.5 b | 25.0 c |
| Root Tissue | 97.4 bc | 136.8 ab | 103.5 b | 46.0 b | 78.0 b | 92.1 b |
| Fern Tissue | 150.0 a | 150.0 a | 150.0 a | 150.0 a | 133.2 a | 140.0 a |
| Asparagus | 72.2 bc | 83.4 c | 18.4 c | 32.5 b | 59.4 b | 28.7 c |

| EXPERIMENT #2 | x$10^6$ CFU/g Soil at the Time of Evaluation (weeks) | | | |
|---|---|---|---|---|
| Treatment | 2 | 5 | 18 | 22 |
| Control | 21.3 b | 30.4 bc | 21.8 c | 11.9 bc |
| Root Tissue | 150.0 a | 143.8 a | 98.6 a | 54.3 a |
| Fern Tissue | 150.0 a | 139.0 a | 122.4 a | 65.6 a |
| Asparagus | 33.8 b | 27.2 bc | 26.4 bc | 24.0 b |
| A. sprengeri | 24.6 b | 35.8 b | 32.4 bc | 14.3 bc |
| Snap Beans | 38.0 b | 23.0 bc | 28.9 bc | 23.4 b |
| Sweet Corn | 18.6 b | 27.2 bc | 67.3 b | 19.5 bc |

*All numbers with a letter in common are not significantly different according to LSD for columns only.

after only 1 week compared to the control soil. Bacterial populations remained high throughout the experiment and at 22 weeks were still 140 x $10^6$ CFU/g soil, whereas control soil populations were 25 x $10^6$ CFU/g soil. Similar increases in the bacterial populations in soil amended with fern tissue were also obtained in the second experiment. As before, root tissue amended soil again showed increased bacterial populations, and the populations began to decline as the experiment progressed. Soil planted with asparagus, A. sprengeri, snap beans, and sweet corn showed no differences in bacterial populations when compared to the control.

The control soil, when considered alone, showed the highest number of fungal CFU after 4 weeks (26.4 x $10^4$ CFU/g soil). In subsequent weeks, population counts were less (Table 2). In pots where an asparagus plant was present in the soil, fungal populations did not vary from those counted in the control soil except at 8 weeks in experiment 1. Fungal populations in treatments where fern tissue was incorporated in the soil were also not different from the control soil except at 8 weeks in experiment 1. However, when root tissue was incorporated in the soil, fungal populations increased significantly over controls after only 1 week. Population counts were always higher in treatments amended with root tissue than in any other treatments.

This increase in fungal CFU's for root tissue also occurred in the second experiment. Fungal populations in soil amended with fern tissue did not differ from controls in either experiment. Fungal populations in soil where snap beans were planted showed a significant increase at 9 weeks as compared to the control soil. Fungal populations in the snap bean treatment at 9 weeks were similar numerically to those in soil amended with root tissue. However, at 18 and 22 weeks, after snap

Table 2: Estimates of fungal populations (CFU's) in Spinks sandy loam soil amended with asparagus root or fern tissue or planted with asparagus, snap beans, or sweet corn. Initial fungal population in 1 g of unamended soil was 9.1 x $10^4$ CFU in Experiment #1, and 6.5 x $10^4$ CFU in Experiment #2.

| EXPERIMENT #1 | x$10^4$ CFU/g Soil at the Time of Evaluation (weeks) | | | | |
|---|---|---|---|---|---|
| Treatment | 1 | 4 | 8 | 12 | 18 |
| Control | 8.0 bcd* | 26.4 bc | 1.4 b | 1.0 b | 7.8 b |
| Root Tissue | 20.3 a | 150.0 a | 88.7 a | 28.3 a | 29.2 a |
| Fern Tissue | 1.3 cd | 55.8 b | 7.0 b | 1.1 b | 3.0 b |
| Asparagus | 8.8 bc | 19.5 c | 38.2 b | 0.8 b | 14.8 ab |
| EXPERIMENT #2 | x$10^4$ CFU/g Soil at the Time of Evaluation (weeks) | | | | |
| Treatment | 2 | 5 | 9 | 18 | 22 |
| Control | 4.0 b | 10.8 b | 7.3 c | 5.8 b | 4.0 b |
| Root Tissue | 83.9 a | 101.1 a | 79.4 a | 46.9 a | 32.4 a |
| Fern Tissue | 18.3 b | 10.4 b | 3.4 c | 3.3 b | 2.6 b |
| Asparagus | 1.5 b | 5.5 b | 3.8 c | 3.3 b | 7.0 b |
| Snap Beans | 2.2 b | 5.8 b | 67.7 ab | 13.8 b | 6.5 b |
| Sweet Corn | 1.2 b | 9.5 b | 31.5 bc | 6.7 b | 6.8 b |

*Numbers without a letter in common are significantly different at P=0.01 or greater according to LSD for columns only.

beans had begun to senesce, populations were similar to levels similar in the control soil. In treatments where asparagus was grown in the soil, populations did not vary from those of the control.

Population counts for Fusarium spp. were significantly higher for treatments where root tissue was incorporated into the soil than in any other treatment (Table 3). This was true for all time periods analysed. All other treatments varied little from control soil. In treatments where asparagus was grown in the soil, Fusarium populations increased significantly from other treatments after 18 weeks, but populations did not reach the levels seen in the root tissue treatments. This result was not obtained in the second experiment in which only the treatment where root tissue was incorporated in the soil showed a significant increase in Fusarium populations.

For actinomycetes, the population counts in all treatments varied considerably and data were not consistent from one experiment to the next. Therefore, no conclusions could be drawn from them.

**Effects of water extracts on isolates of Pythium spp.** Six of the P. ultimum isolates and the one P. aphanidermatum isolate did not grow within 2 cm of the areas where a water extract of asparagus root tissue had been applied on PDA (data not shown). With 3 other isolates, sparse growth of the Pythium hyphae was observed on the area where the root extract was applied. Hyphal strands were appressed on the agar plate and did not exibit the normal fluffy appearance of the rest of the culture or the control plates.

Table 3: Estimates of Fusarium spp. populations (CFU's) in Spinks sandy loam soil amended with dried asparagus root or fern tissue or planted with asparagus, snap beans, or sweet corn. Initial propagule count per 1 g of soil was 20.3 x $10^3$ CFU in both experiments.

| EXPERIMENT #1 | x$10^3$ CFU/g Soil at the Time of Evaluation (weeks) | | | | |
|---|---|---|---|---|---|
| Treatment | 4 | 8 | 12 | 18 | 22 |
| Control | 29.1 b* | 4.9 b | 5.2 b | 2.1 c | 3.6 c |
| Root Tissue | 93.3 a | 79.2 a | 133.4 a | 64.3 a | 67.5 a |
| Fern Tissue | 4.2 b | 6.8 b | 3.1 b | 4.3 c | 6.5 c |
| Asparagus | 4.8 b | 11.0 b | 6.9 b | 26.1 b | 23.4 b |
| EXPERIMENT #2 | x$10^3$ CFU/ g Soil at the Time of Evaluation (weeks) | | | | |
| Treatment | 2 | 5 | 9 | 18 | 22 |
| Control | 13.3 b | 15.8 b | 11.8 b | 10.1 b | 6.5 b |
| Root Tissue | 126.3 a | 82.3 a | 71.8 a | 85.7 a | 79.5 a |
| Fern Tissue | 11.5 b | 5.8 b | 5.0 b | 2.7 b | 0.3 b |
| Asparagus | 7.8 b | 8.2 b | 9.0 b | 9.6 b | 7.8 b |
| Snap Beans | 7.3 b | 12.3 b | 29.8 b | 17.8 b | 4.3 b |
| Sweet Corn | 9.9 b | 18.7 b | 20.6 b | 12.0 b | 11.2 b |

*Numbers with no letters in common are significantly different at P=0.01 or greater by LSD in columns only.

**Sensitivity of bacterial isolates to purified extracts of asparagus root tissue.** Of 18 cultures tested, 8 were inhibited by isolated components of asparagus root tissue eluted from TLC plates (Table 4). Components from rf=0.35 and 0.26 have been shown to also contain compounds inhibitory to seed germination and radical elongation of Lepidium sativum cv. curly cress (6). Components at rf=0.82, 0.75, and 0.5 did not affect seed germination or radical elongation, but were strongly inhibitive of bacterial growth. Isolates of bacteria particularly sensitive to asparagus root extracts were identified as Bacillus spp., and B. circulans.

## DISCUSSION

**Impacts of various tissues and plant species on naturally occuring microbial populations.** In all experiments, the coefficient of variation was high. Data collected in this laboratory and others have shown that high variation is not unusual for microbial population assessments in natural soil. However, despite high variability, statistical analysis suggested significant differences in several cases.

Bacterial species were capable of increasing their populations in soil amended with fern or root tissue to 2-3 fold higher levels when compared to soils planted with asparagus, snap beans, sweet corn, or natural soil. Fern tissue was quickly colonized by bacterial species. Root tissue was also colonized quickly. Decline of the bacterial populations occured more quickly in the root tissue than in the fern tissue. Visual assessment of the samples indicated that the general

Table 4: Inhibition of bacterial isolates by isolated components of asparagus root extracts.

| Isolate #[a] | Sensitive Microorganisms | Rf[b] of Active Fractions |
|---|---|---|
| 2a | Bacillus spp. | 0.35 |
| 3a | B. circulans | 0.82 |
| | | 0.80 |
| | | 0.26 |
| 7 | B. spp | 0.35 |
| 7a | B. circulans | 0.26 |
| 10 | B. circulans | 0.35 |
| 16 | B. circulans | 0.35 |
| 17 | B. circulans | 0.35 |
| 48 | B. circulans | 0.82 |
| | | 0.75 |
| | | 0.50 |
| | | 0.35 |

[a]Isolates collected from Spinks sandy loam soil never before under cultivation.

[b]Rf of purified asparagus root component inhibitory to bacterial growth. Components were eluted from TLC silica gel plate (developed with hexane:ethyl acetate 1:1 as the mobile phase) with methanol or chloroform.

species diversity in the soil had changed in both root and fern tissue treatments. This indicated that tissues contained compounds that selectively inhibited some bacteria or that certain species of bacteria were better able to colonize the root and fern tissue than others. Since root and fern tissue had been surface sterilized before amending into soil, the change in species population was not caused by contaminating bacterial species on the dried tissues.

Both total fungal and Fusarium spp. population counts increased in number when root tissue was incorporated into soil. Fusarium populations increased quickly in the soil and remained at significantly high levels throughout the duration of the experiments. Although total fungal populations increased, counts began to decline after 4 weeks in the first experiment and after 5 weeks in the second experiment. In asparagus plant treatments, fungal populations were observed at levels similar to those in the control soil. Fungal populations in the snap bean and sweet corn treatments initially increased, then declined over time. These data agree with observations of fungal populations in asparagus plantations, snap bean fields, and corn fields in agroecosystems in Michigan (unpublished data). These data may suggest that competitive saphrophytic fungal species may be incapable of developing in the rhizosphere of the asparagus plant.

**Effects of water extracts on isolates of Pythium spp.** Water extracts of asparagus root tissue were capable of inhibiting completely or depressing hyphal growth of isolates of Pythium spp. These data suggest that certain soil borne fungal species are sensitive to the compounds contained in asparagus root extracts, and that these

compounds may be affecting the species diversity in soils with an excess of asparagus root tissue.

**Sensitivity of bacterial isolates to purified extracts of asparagus root tissue.** One third of the bacterial isolates collected from sandy soil were inhibited by purified extracts from asparagus root tissue. These data suggest that isolated toxins from asparagus root tissues are capable of reducing the diversity of saphrophytic soil bacteria.

When compared to other plant species, fungal populations are not as high when asparagus plants are grown in the soil. However, fungal populations, particularly Fusarium can increase suprisingly quickly on asparagus root tissues, but not on asparagus fern tissues. Bacteria colonize fern tissue very quickly and populations remain high in soil where fern residue is present. This may suggest bacteria quickly break down fern tissue, but not root tissue. Colonization and subsequent breakdown of the fern tissue by bacteria may account for the absense of toxicity in nonsterile soil reported in the literature. Data presented here suggest that toxins present in asparagus root tissues are capable of reducing the numbers and diversity of microbial populations in natural soil. Since the herbaceous perennial asparagus plant is always producing new tissues and older tissues are continually dying in the soil, there is always a substantial amount of tissue in various stages of development in the soil system. Data in these experiments suggest fungal population and Fusarium populations may increase in a relatively short time on excess tissue. Other microorganisms may be detrimentally affected also. This phenomenon may contribute to some of the problems associated with asparagus decline in asparagus plantations.

## LITERATURE CITED


1. Cohen, Sylvan. I. and F.D. Heald. 1941. A wilt and root rot of asparagus caused by Fusarium oxysporum Schlecht. Plant Disease Reporter. 25: 503-509.
2. Endo, R. M., and E. C. Burkholder. 1971. The association of Fusarium moniliforme with the crown rot complex of asparagus. Phytopathol. 61: 891 (Abstr.)
3. Fischer. H-P., and D. Bellus. 1983. Phytotoxicants from microorganisms and related compounds. Pestic. Sci. 14: 334-346.
4. Grogan, R. G., and K. A. Kimble. 1959. The association of Fusarium wilt with the asparagus decline and replant problem in California. Phytopathol. 49: 122-125.
5. Johnson, S.A., J.K. Springer, G.D. Lewis. 1979. Fusarium moniliforme as a sourse of stem and crown rot of asparagus and its association with asparagus decline. Phytopathol. 67:778-780.
6. Hartung, A. C., and C. T. Stephens. 1983. Effects of allelopathic substances produced by asparagus on incidence and severity of asparagus decline due to Fusarium crown rot. J. Chem. Ecol. 9:1163-1174.
7. Hartung, A. C., and A. R. Putnam. Extracts of asparagus root tissue are phytotoxic. Proceedings of the Sixth International Asparagus Symposium. E. C. Lockheed and H. Tiessen, eds. Univ. of Guelph. pp. 258-266.

8. Komada, H. 1975. Development of a selective medium for quantitative isolation of Fusarium oxysporum from natural soils. Rev. Plant. Plot Res. 8: 114-125.

9. Laufer, G. A., and S. A. Garrison. 1977. The effect of asparagus tissue on seed germination and asparagus seedling growth. Possible allelopathic interaction. HortSci. 12:385 (Abstr.)

10. Lehle, F. R. and A. R. Putnam. 1982. Quantification of allelopathic potential of sorghum residues by novel indexing of Richards Function fitted to cumulative cress seed germinations. Plant Physiol. 69:1212-1216.

11. Lockwood, J. L. 1968. The fungal environment of soil bacteria. In: The Ecology of Soil Bacteria, an International Symposium. T. R. G. Gray and D. Parkinson, eds. Liverpool Univ. Press. pp. 65.

12. Linderman, R. G. 1970. Plant residue decomposition products and their effects on host roots and fungi pathogenic to roots. Phytopathol. 60:19-22.

13. Martin, J. K. 1975. Comparison of agar media for counts of viable soil bacteria. Soil Biol. Biochem. 7:401-402.

14. McCalla, T. M., and F. A. Haskins. 1961. Phytotoxic substances from soil microorganisms and crop residues. Bact. Rev. 28:181-207.

15. Patrick, Z. A., 1971. Phytotoxic substances associated with the decomposition in soil of plant residues. Soil Sci. 13-18.

16. Patrick, Z. A., and L. W. Koch. 1963. The adverse influence of phytotoxic substances from decomposing plant residues on resistance of tobacco to black root rot. Can. J. Bot. 41:746-758.

17. Proebstring, E. L., and A. E. Gilmore. 1941. The relation of peach root toxicity to the re-establishment of peach orchards. Proc. Am. Soc. Hort. Sci. 38:21-26.

18. Rice, E. L. 1984. Allelopathy. Academic Press. N.Y. Pp.422.

19. Rice, E. L. 1979. Allelopathy-An Update. Bot. Rev. 45:15-109.

20. Shafer, W. E., and S. A. Garrison. 1980. Effects of decomposing asparagus root tissues on lettuce, tomato, and asparagus seed emergence. HortSci. 15:406. (Abstr.).

21. Shafer, W. E., and S. A. Garrison. 1980. Effects of aspargus root extracts on lettuce and asparagus seed germination and growth. HortSci. 15:406-407. (Abstr.).

22. Shafer, W. E., and S. A. Garrison. 1986. Allelopathic effects of soil incorporated asparagus roots on lettuce, tomato, and asparagus seedling emergence. HortSci. 21(1):82-84.

23. Takatori, F., and F. Souther. 1978. Asparagus Workshop Proceedings. Dept. Plant Sciences, University California, Riverside, CA. pp 156.

23. Toussoun, T. A., and Z. A. Patrick. 1963. Effect of phytotoxic substances from decomposing plant residues on root rot of bean. Phytopathol. 53:265-270.

24. Toussoun, T. A., A. R. Weinhold, R. G. Linderman, and Z. A. Patick. 1968. Nature of phytotoxic substances produced during plant residue decomposition in soil. Phytopathol. 58:41-45.

25. Yang, Hsu-Jen. 1982. Autotoxicity of Asparagus officinalis L. J. Amer. Soc. Hort. Sci. 107:860-862.

26. Young, C. C. 1984. Autointoxication in root exudates of Asparagus officinalis L. Plant and Soil. 82:247-253.

27. Young, C. C. 1986. Autointoxication of *Asparagus officinalis* L. in, *The Science of Allelopathy*. A. R. Putnam and C. S. Tang, eds. John Wiley and Sons, Inc. pp 317.

# CHAPTER V

## ISOLATION AND CHARACTERIZATION OF ALLELOCHEMICALS IN ASPARAGUS (*ASPARAGUS OFFICINALIS* L.) ROOT TISSUE


## ABSTRACT

Isolation and characterization of allelochemicals from asparagus (*Asparagus officinalis* L.) were attempted using aqueous extracts of aspragus root tissues. Ferulic, isoferulic, malic, citric, and fumaric acids were all found to be present in phytotoxic fractions isolated by HPLC. These acids were active in Curly Cress (*Lepidium sativum*) bioassays with $I_{50}$ levels of 0.5 mg/ml for ferulic, 1.4 mg/ml for malic, and 1.6 mg/ml for citric. Isoferulic acid gave no inhibition of curly cress at any level tested. Bioassays comparing activities of combined standards and crude extracts at different pH levels showed that inhibitory activity of known standards and crude extracts was not affected by pH. NMR spectra suggested that a derivative of asparagusic acid or asparagusic acid 3-oxide was present in an inhibitory fraction isolated from preparative TLC plates. Isolation of inhibitory compounds using soxhlet extraction of dried asparagus root tissue revealed caffeic acid to be an additional phytotoxin in the root tissue. However, bioassays comparing the inhibitory activity of a caffeic acid standard with the crude caffeic acid fraction showed pure

caffeic acid to be less active ($I_{50}$=2.0 mg/ml) than the crude fraction ($I_{50}$=0.26 mg/ml). This suggested that a derivative of caffeic acid might be involved.

## INTRODUCTION

Asparagus (Asparagus officinalis L.) is a perennial vegetable crop grown on sandy soils in temperate areas of the Eastern and Western United States. Asparagus decline, resulting in reductions in commercial yields and quality, and death of crowns in fields continuously cropped to asparagus is a well known and well documented problem on asparagus in many production areas (2, 4, 8, 7). Asparagus decline is ultimately attributed to the presence of two soil-borne pathogens, Fusarium oxysporum (Schlecht. ) emend. Snyd. and Hans. f. sp. asparagi Cohen, and F. moniliforme (Sheld.) emend. Snyd. and Hans., that once introduced to ecosystem, survive in the soil indefinitely (1, 2, 4, 9). However, other laboratories as well as this one, have presented evidence that asparagus is an autotoxic or autoallelopathic plant (5, 6, 7, 11, 16, 17, 18, 20, 22). Allelochemicals are defined as compounds released from one plant (or microorganisms) that affect the growth of another plant (14). Autotoxicities are considered as special cases of allelopathy, and the same compounds may exert interspecies effects. Compounds released from living or senescing asparagus root tissue are hypothesized to damage the asparagus root system, which may decrease the growth of the crown (11, 16, 17, 18, 20, 22, 23). Injury to the root system from these autotoxic compounds may also provide infection courts for the entrance of the Fusarium

pathogens, therefore enhancing the incidence of disease on the plant (5). Young (23) has reported that several phenolic compounds are released from the root system of intact asparagus plants. Using an XAD-4 resin trapping procedure originally developed to recover phenolic and chlorophenoxy herbicides from soil, he isolated 3,4-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dimethoxyacetophenone, and β-(m-hydroxyphenyl)propionic acid. However, he did not report if these compounds were inhibitory to asparagus seedlings nor did he examine if senescing tissue possessed any inhibitory compounds that may also be contributing to the damage in the field. He also did not determine if these compounds were of plant or microbial origin. Kitahara, et. al. (10) isolated three compounds they called growth regulators from etiolated asparagus ferns, asparagusic acid (1,2-dithiolane-4-carboxylic acid) with activity comparable to abscisic acid, dihydroasparagusic acid (β,β'-dimercaptoisobutyric acid), and S-acetyldihydroasparagusic acid (β-S-acetyl-β'-mercaptoisobutyric acid) (Figure 1). Though these compounds are considerably toxic to seedling germination and radical elongation of several seed species, these compounds occurred at extremely low concentrations in the tissues and are quite labile once isolated. Asparagusic acid has also been isolated from the roots of asparagus and found to be inhibitory to nematodes, specifically the emergence of the second stage larve of Heterodera rostochiensis and H. glycines and the second stage larvae of H. rostochiensis and Meloidogyne hapla, and the larvae and adults of Pratylenchus penetrans and Paratylenchus curvitatus (19). A patent is presently held by Sankyo Co. Ltd., Japan, on asparagusic acid and its derivatives for activity on nematodes and plant growth regulation.

Since asparagus possesses large storage roots that are continually dying as the crown grows, large amounts of asparagus root material may be present in asparagus fields at any one time. It has been reported that these root residues contain a considerable amount of toxic substances that inhibit seed germination and radical elongation of different seedling species (16, 17, 18), as well as asparagus growth, peroxidase activity of asparagus seedlings, and respiration of the crown (7). At this point, no one has isolated compounds that can account for all the inhibitory activities present in the root tissue. Also, even though several compounds have been isolated from asparagus tissues, none have been isolated from or shown to be active in the agroecosystems where asparagus is grown. The purpose of this research was to attempt to isolate compound(s) from asparagus root tissue that are responsible for the reported allelopathic properties of asparagus and test their effectiveness in bioassays, and to try and recover these compounds from the soil.

## MATERIALS AND METHODS

**Preparation of asparagus plant material.** Asparagus crowns (cv. Martha Washington) were excavated from a research plot (7 yr old) located at the Horticultural Research Farm, Michigan State University, E. Lansing, MI. The storage and fibrous roots were separated from the rhizomes and all dead and visibly diseased plant material was discarded. The roots were oven dried at 50 C and ground in a Wiley Mill (mesh screen size=1 mm). All plant tissues were sterilized with propylene oxide gas and checked for microbial contamination as previously reported (6, 7).

Containers with sterilized root tissue were allowed to exhaust under a fume hood for 24 hr to dissipate any residual propylene dioxide. Dried sterilized tissue was stored in brown glass bottles at -20 C until used for chemical isolation.

**Bioassay procedure for evaluation of isolated components of asparagus root extractions.** Previous studies in this laboratory have shown that Lepidium sativum L. (cv. Curly Cress) is a fast and reliable seedling for evaluating the inhibitory activity of many allelopathic compounds (12). Therefore, curly cress was used to test for inhibitory activity from root extracts throughout each step of all isolation procedures and for evaluation of activity from known standards of isolated compounds. All fractions to be assayed were first taken to dryness and weighed. Separate fractions were then redissolved in methanol (or an appropriate solvent, depending on solubility of the fraction) and a dilution series was made. A known amount of each fraction was spotted on filter paper (Whatman #1) in glass petri dishes. Evaporated solvent controls and $DH_2O$ (deionized water) controls were always included in each bioassay. Also, crude extract of asparagus root often included as a control when appropriate. All fractions were filter sterilized before bioassaying. The solvent was allowed to evaporate completely from the filter paper and 1.5 ml of $DH_2O$ was added to the dish. Curly cress seeds (10/dish) were added to the dish and distributed uniformly with a glass rod. The dishes were covered, arranged in a randomized complete block design, and placed in a moist chamber for 72 hr at 26 C. Root length was measured and $I_{50}$ levels determined by interpolation. There were 3 replications per treatment in all cases except when paucity of the

compound only allowed for 2 replications or single observations. When replication was impossible, a standard error was calculated for the root length of individual seedlings. When there was replication, the data were subjected to an Analysis of Variance and the means separated by the LSD test.

**Bioassays of known compounds and crude extracts.** When available, standards of isolated compounds were tested for inhibitory activity and compared to those of the crude inhibitory fractions from the C18 column. Known standards were bioassayed from 0.025 ug/ml to 2.0 mg/ml concentrations.

Bioassays comparing the known standards and the active fractions from the C18 column at different pH levels were done to assure that inhibition seen in the fractions was not simply a pH effect. The C18 fractions and the known standards were bioassayed at their original pH as well as the pH of the other fraction. The standards were combined, the pH determined (pH=3.6), then the solution was separated into two treatments; one was left at the original pH and the other adjusted to the pH of the C18 fraction (pH=5.6). The same procedure was done with the C18 fraction. The combined standards and the crude were bioassayed at 125, 250, and 500 ppm. The C18 fraction was also bioassayed at pH 7.0 at 25, 50, and 150 ppm. A buffer control and methanol solvent control were also included in the bioassay.

**Purification of inhibitory components in asparagus root extracts: Scheme #1.** Previously published data from several laboratories and this one have indicated that water extracts of asparagus root tissues are inhibitory to seedling germination and radical elongation (6, 11,

17). Therefore water extracts from asparagus root tissue were subjected to sequential extraction with solvents to purify the chemical components implicated in the inhibitory responses. Ground asparagus root tissue was extracted by stirring overnight at 4 C (1:10, root tissue:$DH_2O$) (Figure 2). The particulates were removed by filtering through four layers of cheese cloth and centrifuging at 6,000 x g for 20 min. The supernatant was decanted carefully from the pellet (the pellet was discarded) and then proteinaceous and lipophyllic materials precipitated with acetone (4:1 v:v acetone:sample) by slowly stirring overnight at 4 C. The precipitate was removed from the eluant by filtering through filter paper (Whatman #4, #1, and #42 respectively) and bioassayed. The precipitate was discarded and the liquid concentrated to 1/4 the original volumn on a Buchi Rotary evaporator at 40 C. This concentrate was sequentially extracted with the following solvent series: chloroform, diethyl ether, dichloromethane, ethyl acetate and water-saturated n-butanol. The aqueous root extract was partitioned three times (1:1 v:v), with each of the six solvents. Each solvent fraction was dried with $MgSO_4$, concentrated to dryness by rotary evaporation at 40 C, weighed and bioassayed at 10 and 100 ppm. The data were subjected to an Analysis of Variance and means separated by the LSD test. The percent of the original fraction represented by each solvent extraction was calculated.

The chloroform fraction was further purified by column chromatography (Figure 3). The dried chloroform fraction was first redissolved in methanol, after which a white precipitate formed. The precipitate was separated from the methanol soluble supernatant by filtering through filter paper (Whatman #4, #1, and #42 respectively)

Figure 2. Flow diagram for the extraction and partitioning of dried asparagus root tissue.

## EXTRACTION PROCEDURE

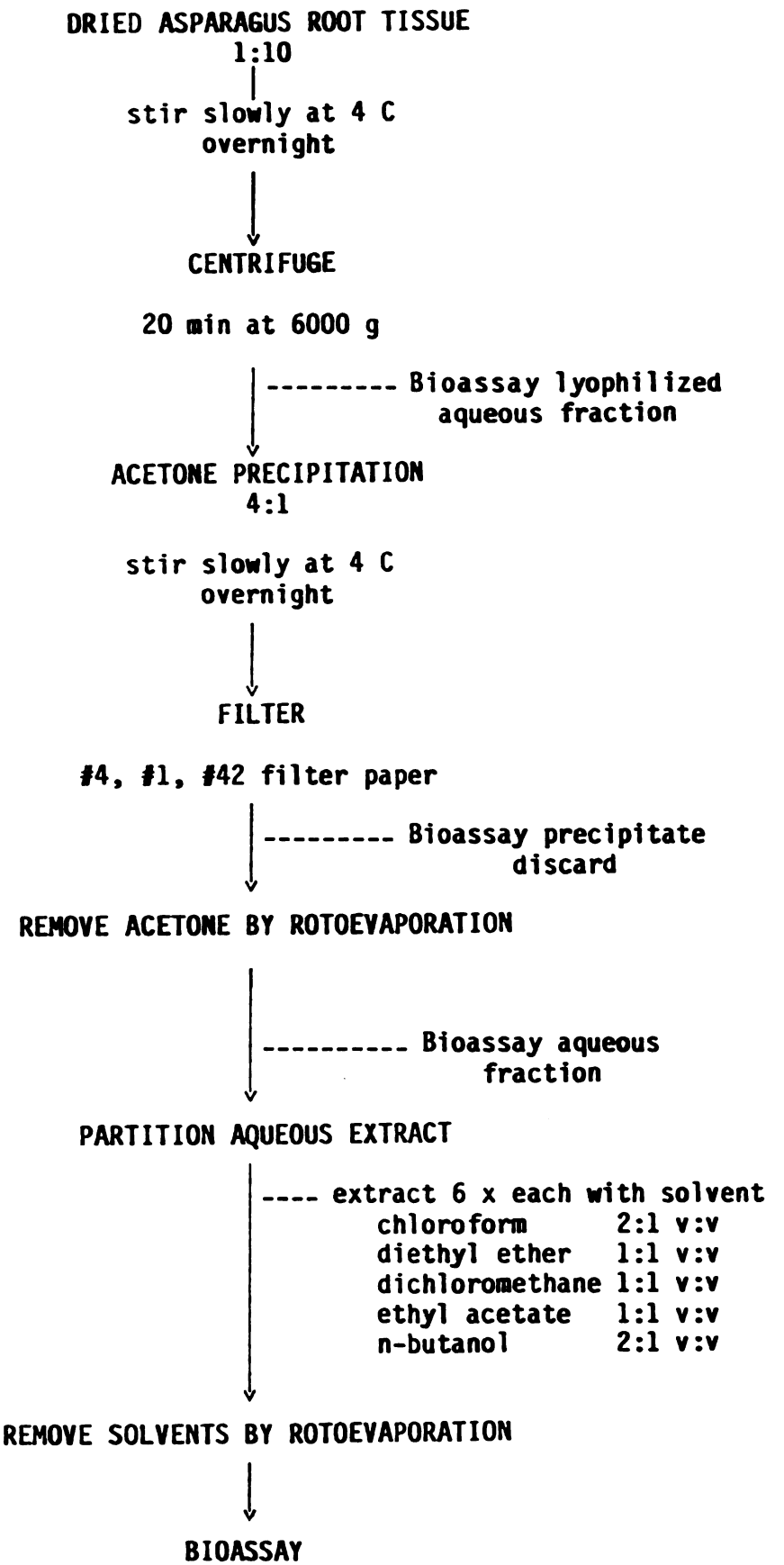
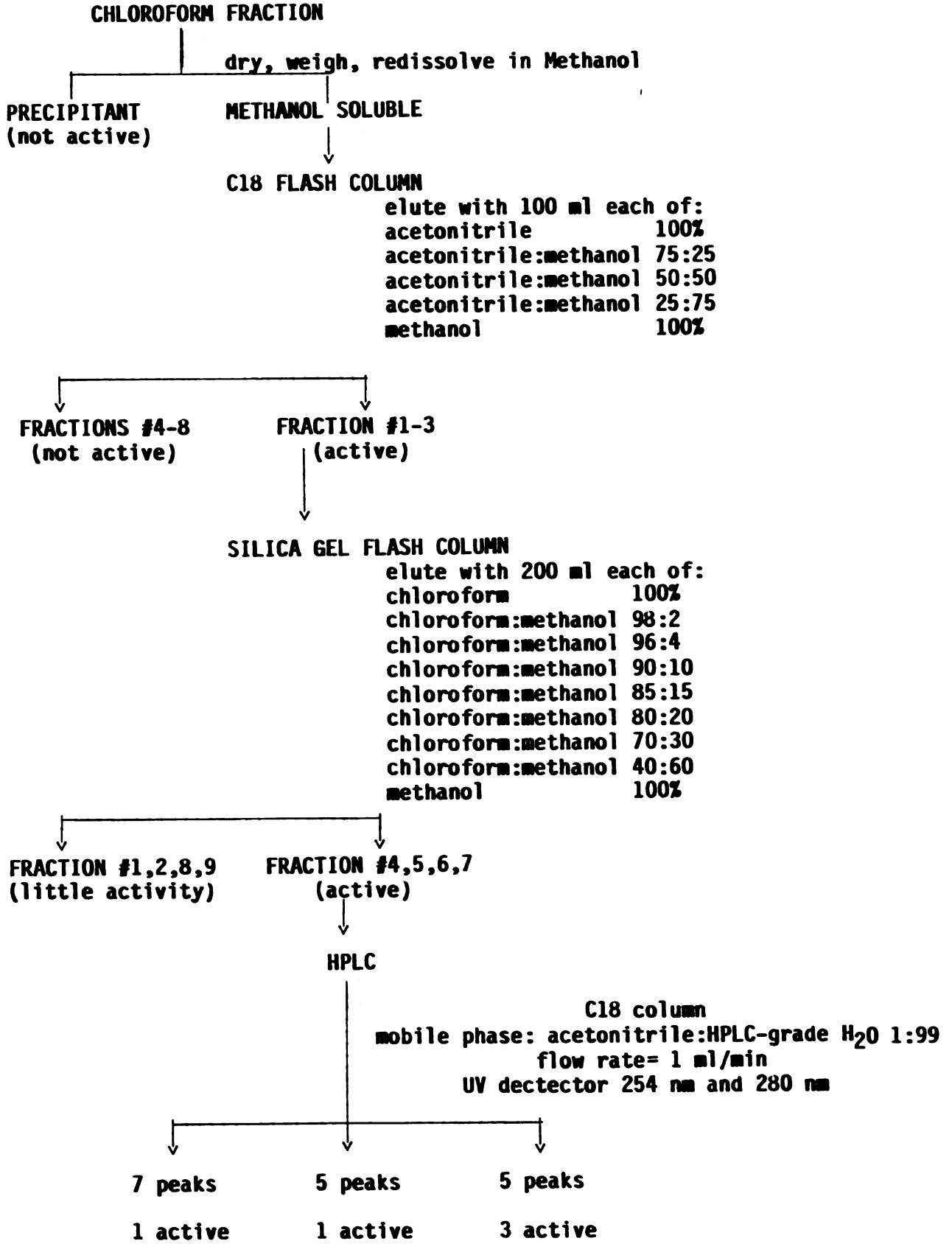


Figure 3. Flow diagram for the chromatographic separation of compounds from the chloroform extract of dried asparagus root tissue.

## COLUMN CHROMATOGRAPHY OF CHLOROFORM FRACTION



in a buchner funnel. The methanol soluble fraction was further purified using an octadecyl (C18) bonded phase solid support in a Baker flash chromatography column (190 mm x 20 mm) eluted with a step gradient of acetonitrile (100%) to methanol (100%) at 25% intermediate steps in 100 ml fractions. Pressure for the column was provided by laboratory air line at a rate of 0.2 cm/sec. Fractions (25 ml) from the column were collected, spotted on thin layer chromatography plates: (Whatman silica gel 60 F-254; mobile phase: chloroform:methanol 9:1 and chloroform:methanol 8:2), then recombined according to similar Rf values. The fractions were dried under nitrogen, weighed, and bioassayed at 25, 50, and 100 ppm. The first three fractions were combined and separated further on a Baker flash chromatography column (180 x 30 mm) using a silica gel solid support and a mobile phase gradient of chloroform (100%) to methanol (100%) in the following order: 100% chloroform, 98:2, 96:4, 90:10, 85:15, 80:20, 70:30, 40:60, 100% methanol. Each gradient step was applied to the column in 200 ml fractions. Air pressure for the column was as above. Fractions of 75 ml each were collected, concentrated, and developed on TLC plates as above. Fractions with similar Rf values were combined, dried under nitrogen, weighed, and bioassayed at 25 and 50 ppm. Individual fractions were then further purified by high pressure liquid chromatography (HPLC) using a Waters uBondapak C18, 8 mm x 10 cm radial compression column. The UV detector (Waters 490 multiwave programmable) measured absorbance at 254 and 280 nm. The solvent system was acetonitrile:$H_2O$, 1:99 at a flow rate of 1.0 ml/min. The mixture was resolved into 5 distinct areas and 5 fractions were collected after repeated injections. Three separate fractions from the silica gel flash columns were further separated in this manner. Each

fraction from the HPLC collection was dried under nitrogen, weighed, and bioassayed at 50 ppm on curly cress. From each of these HPLC separations, purified samples were sent to Dr. Basil Burke at Arco Plant Cell Research Institute, Dublin, CA, for spectral evaluation. HPLC fractions were silylated and then evaluated by GC-Mass Spectroscopy (GC-MS) using a temperature gradient from 110-250 C at 15 degrees/min. Spectral scans from total ion chromatogram were observed and a library search done on each spectrum using the Finnigan NBS Library Compilation series. Standards of elucidated compounds were then injected into the GC-MS ($15^{o}$/min on a 30 M DBS column) under the same conditions to further substantiate the identification of these compounds by matching GC retention times, as well as comparing Mass Spectra.

**Purification of inhibitory compounds in dried asparagus root tissue: Scheme #2.** The methanol soluble portion of the chloroform fraction isolated as above was also separated using preparative TLC. Silica gel (Merck DC-Fertigplatten, 60 F-254 0.5 mm, 20 x 20 cm) plates were first washed in 85:15 chloroform:methanol, then approximately 600 mg of the methanol soluble chloroform fraction was streaked near the base of the plate. In all, 6 plates were used. Plates were run twice then viewed under UV (254 nm) light. Nine distinct bands were scraped off the silica plates and eluted from the silica gel using chloroform 100%, (or chloroform:methanol, 5:1 v:v for bands at Rf>0.4) through a fritted glass filter and collected, dried, weighed, and bioassayed at 30 ppm. One fraction (Rf=0.53) was rechromatographed on silica gel plates as above. Again nine fractions were detected by UV (254 nm) light,

scraped and eluted from the plates as above. A portion of five of these fractions (Rf=0.69, 0.61, 0.52, 0.46, 0.29, and 0.07) were silylated for GC-MS analysis and the $^{1}H$ NMR spectrum of remaining portion of each of these small fractions were obtained deuterated methanol on a Varian XL 300 MHz nuclear magnetic resonance spectrometer (NMR).

**Purification of inhibitory components from dried asparagus root tissue: Scheme #3.** Dried asparagus root tissue (250 g) was extracted with hexane for 4 hr using a soxhlet extraction device. The hexane was removed and replaced with methanol and the tissue further extracted overnight with methanol. The methanol extract was then acidified by dilute HCl to pH 2.0 and extracted with 50 ml of ether 5 times. This ether fraction was then extracted with 75 ml of 5% aqueous sodium bicarbonate 2 times. The alkaline portion was then acidified with HCl to pH 2.0, then extracted with 50 ml of ether 3 times. Fractions were then visualized on TLC after development with mobile phase; chloroform:methanol 8.5:1.5, and mobile phase; toluene:ethyl formate:formic acid, 5:4:1). In the last ether fraction, a yellow compound crystallized on the sides of a round bottomed flask. These crystals were removed by decanting the ether from the flask, washing the crystals with hexane, then dissolving the crystals in methanol. The crystals were prepared with deuterated methanol for structural elucidation on the NMR.

All fractions from this isolation procedure were bioassayed on curly cress. The data was subjected to an Analysis of Variance and the means separated by LSD. A dilution series of the last ether fraction was bioassayed at 0.025-2.0 mg/ml using curly cress.

**Isolation of inhibitory components from soil.** Soil was collected from the rhizosphere of 20 and 40 year-old commercial asparagus fields where asparagus decline had been observed in Oceana County, MI, as well as adjoining control fields where no asparagus was grown. There was no possibility of water runoff from the adjacent asparagus field into the control area, and the soil type was essentially the same as in the asparagus field. Soil was sieved through a 2000 μ screen to remove most plant fragments. Soil (4.5 kg) was extracted with Methanol:$DH_2O$ overnight at 4 C. Soil particulates were removed from the methanol:water solution and the methanol removed from the aqueous fraction by rotoevaporation. Precipitation with acetone and subsequent removal and extraction with chloroform was done on the soil extract as described in the procedure for asparagus tissue extraction. The chloroform soluble fraction was concentrated, dried under nitrogen, weighed and bioassayed on curly cress as described above at concentrations ranging from 32.7 mg/ml to .032 mg/ml. There were three replications per treatment and the data were subjected to Analysis of Variance.

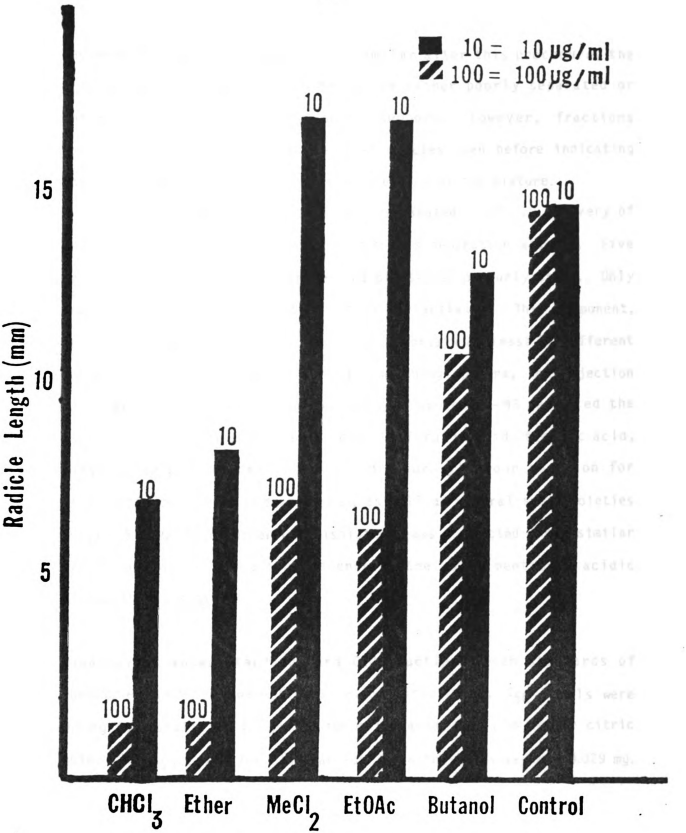
## RESULTS

**Purification of inhibitory components in asparagus root extracts: Scheme #1.** Water extraction of dried asparagus root tissue extracted approximately 35% of the mass from the original material. After acetone precipitation, approximately 83% of the water extract remained. Chloroform extraction accounted for 0.022%, diethyl ether, 0.009%, dichloromethane, 0.004%, ethyl acetate, 0.011%, and n-butanol, 0.12% of

the original dried root material. When bioassayed on curly cress, the chloroform fraction contained the highest specific activity ( $I_{50}$=17.5 ppm in this extraction) (Figure 4). The diethyl ether fraction also had considerable activity ($I_{50}$=34 ppm). Dichloromethane and ethyl acetate fractions also contained inhibitory activity but to a lesser extent than the chloroform and ether fractions ($I_{50}$=82 and 100 ppm respectively). Also, at lower concentrations, the dichloromethane and ethyl acetate fractions were slightly stimulatory. Because of the greatest inhibitory activity in the chloroform fraction, this fraction was chosen for further isolation attempts. By refining the isolation technique, chloroform sometimes removed as much as 0.17% of the original dried material, but based on a weight for weight basis, $I_{50}$ levels were aways very similar, usually being less than 40 ppm for each isolation.

Fractionation of the methanol-soluble portion of the chloroform fraction on an octadecyl bonded phase soild support flash column and subsequent bioassay showed the first 3 fractions to be inhibitory ($I_{50}$=25 ppm). TLC indicated that these fractions were chemically similar and they were therefore combined. However, these active fractions still contained several chemical components and were further fractionated on a silica gel solid support flash column. Nine fractions were collected, 4 of which gave 98% inhibition of curly cress at less than 50 ppm. Fraction #1 and #2 contained some inhibitory activity at 100 ppm but had no Rf values in common when developed on TLC plates. When developed on TLC, the four inhibitory fractions showed only one common Rf area so were kept separate. $I_{50}$ levels for these fractions were 35 ppm for fraction #4, 58 ppm for fraction #5, 18 ppm for fraction #6, and 30 ppm for fraction #7.

Figure 4. Response of curly cress to 10 and 100 µg/ml of solvent extracts from aqueous extract of dried asparagus root tissue.


10 = 10 µg/ml
100 = 100 µg/ml
Radicle Length (mm)
15
10
5
10
100
10
100
10
100
10
100
10
100
10
100
10
CHCl3
Ether
MeCl2
EtOAc
Butanol
Control
Solvent Extraction of Root Tissue

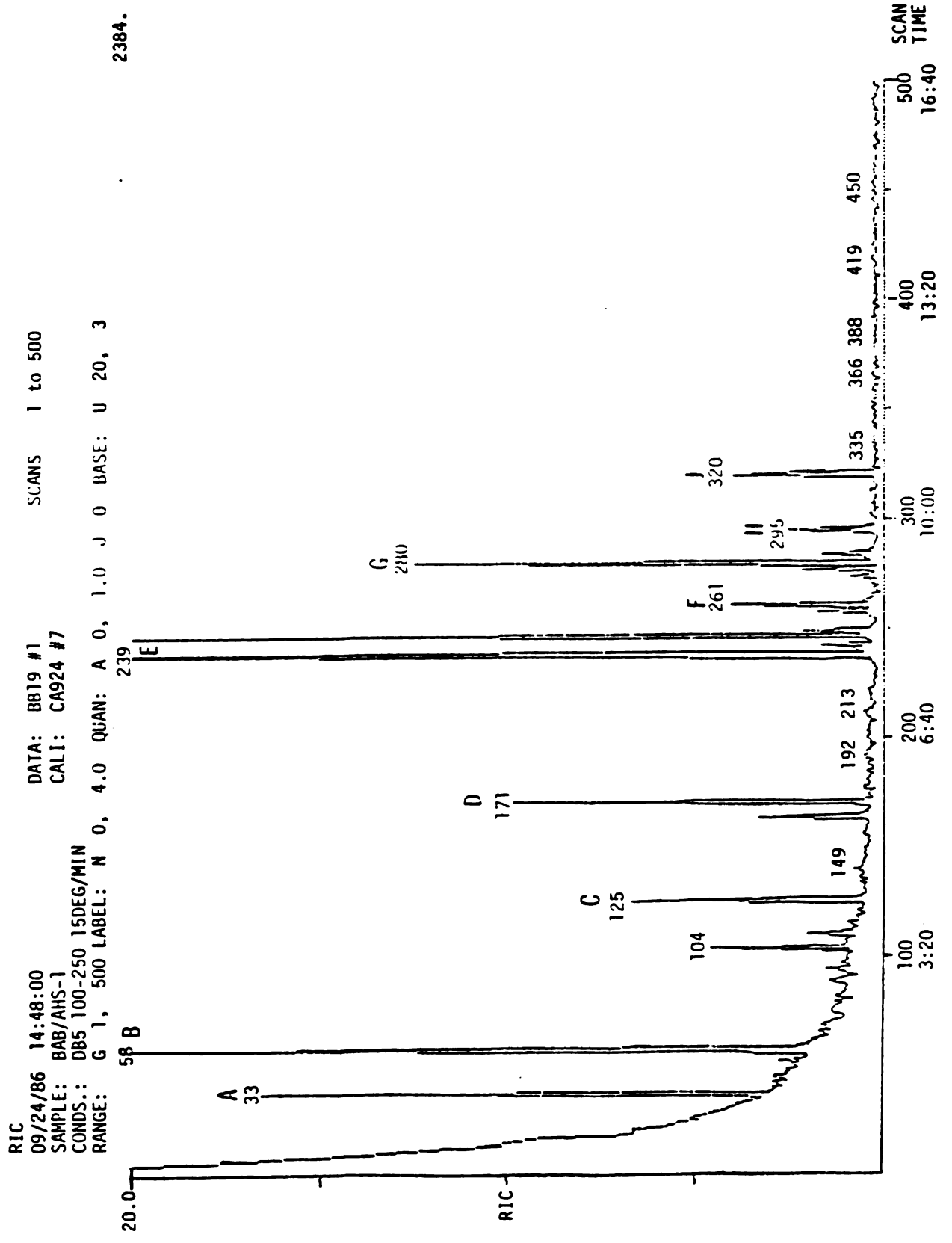
Because the $I_{50}$ levels were quite similar after this procedure, the active components were suspected to be either poorly separated or interacting with the column solid support. However, fractions contained substantially fewer chemical species than before indicating that the silica column provided some clean-up of the mixture.

The components in fraction #6 were separated on HPLC. Recovery of dry weight from repeated injections for this separation was 67%. Five separate peak areas were collected and bioassayed on curly cress. Only one peak area proved to contain inhibitory activity. This component, when evaluated by GC-MS still appeared to contain at least 12 different compounds. A library search done for matching spectra, and injection of known standards at the same conditions on the GC-MS revealed the presence of fumaric acid, malic acid, isoferulic acid, ferulic acid, dihydrocitric acid, and citric acid (hydroxyl group position for citric acid could not be determined), as well as several sugar moieties (Figure 5). GC-MS of other HPLC isolated peaks collected under similar conditions also showed the presence of the above mentioned acidic compounds and sugars.

**Bioassay of known standards and C18 fraction.** When standards of identified compounds were bioassayed on curly cress, $I_{50}$ levels were 0.5 mg for ferulic acid, 1.46 mg for malic acid, and 1.58 mg for citric acid. The $I_{50}$ level for C18 fraction #1 in this bioassay was 0.029 mg.

There was no difference in the activity seen at pH=5.6 or 3.5 in the crude fraction ($I_{50}$ for both treatments=42 ppm). The combined standards only inhibited curly cress at the 500 ppm level. Inhibition for the standards was only significant at P=0.05 and no $I_{50}$ could be

Figure 5. Total ion current of GC-MS of Fraction #3 of peak collected from HPLC separation of dried asparagus root extract tha was inhibitory to cress seed germination and radicle elongation. A, B = silylated peaks; C = Fumaric acid; D = Malic acid; E = Dihydro citric; F = Mannose; G = Isomer of ferulic acid; H = Glucopyranose; and J = Ferulic acid or isoferulic acid.


RIC
09/24/86 14:48:00
SAMPLE: BAB/AHS-1
CONDS.: DB5 100-250 15DEG/MIN
RANGE: G 1, 500 LABEL: N 0, 4.0 QUAN: A 0, 1.0 J 0 BASE: U 20, 3
DATA: BB19 #1
CALI: CA924 #7
SCANS 1 to 500
2384.
20.0
RIC
A 33
B 58
C 125
D 171
E 239
F 261
G 280
H 295
J 320
104
149
192
213
335
366
388
419
450
100 3:20
200 6:40
300 10:00
400 13:20
500 16:40
SCAN TIME

determined.

There was slight inhibition of the curly cress at the higher concentrations of the phosphate buffer control in bioassays comparing the C18 extract at pH=5.6 and 7.0. Therefore, the evaporated methanol control may be a more appropriate benchmark. Using the methanol solvent control, the $I_{50}$ level, the $I_{50}$ for the crude was 20 ppm.

**Purification of inhibitory compounds in dried asparagus root tissue: Scheme #2.** When the compound at Rf=0.07 was subjected to $^{1}H$ NMR in deuterated methanol, the resulting spectrum suggested the structure may be that of asparagusic acid-1-oxide (1,2-dithiolane-4-carboxylic acid-1-oxide) or asparagusic acid (1,2-dithiolane-4-carboxylic acid) (Figure 6,1). The spectrum also reveals several signals representing impurities associated with the methods used. The singlet at $\delta$ 1.22 represents a co-eluate from TLC adsorbents, an artifact usually observed when small quantities are purified by preparative TLC. Singlets at $\delta$ 3.22 and 4.80 correspond to undeuterated methanol, partially deuterated water, and the exchange of the OH group on the acid moiety of the compound. The spectrum also shows a clear multiplet at $\delta$ 3.98-4.12 ( 1 H, m) that is attributed to $H_3$, an absorption at $\delta$ 3.82-3.87 (1 H, dd, J=8 Hz, J=6 Hz), corresponding to $H_4$, absorption at $\delta$ 3.6-3.66 (2 H, J values unable to be determined) corresponding to $H_2$ and $H_5$, and absorption at $\delta$ 3.45-3.54 (1 H, t J=6 Hz) attributed to $H_1$ (Figure 1). These values agree well with those published by Yanagawa (21), except for a slight downward shift of the ppm values. This downward shift is probably due to the difference in solvent use to dissolve the fraction in, and the difference in the type of NMR used to evaluate the sample. When this compound was silylated, the GC-MS

Figure 6. $^{1}H$ NMR of a compound isolated from asparagus root tissue using preparative TLC, mobile phase, chloroform:methanol 8.5:1.5. The compound at Rf=0.07 was scraped from the plate and eluted from the silica using cholorform:methanol 5:1.

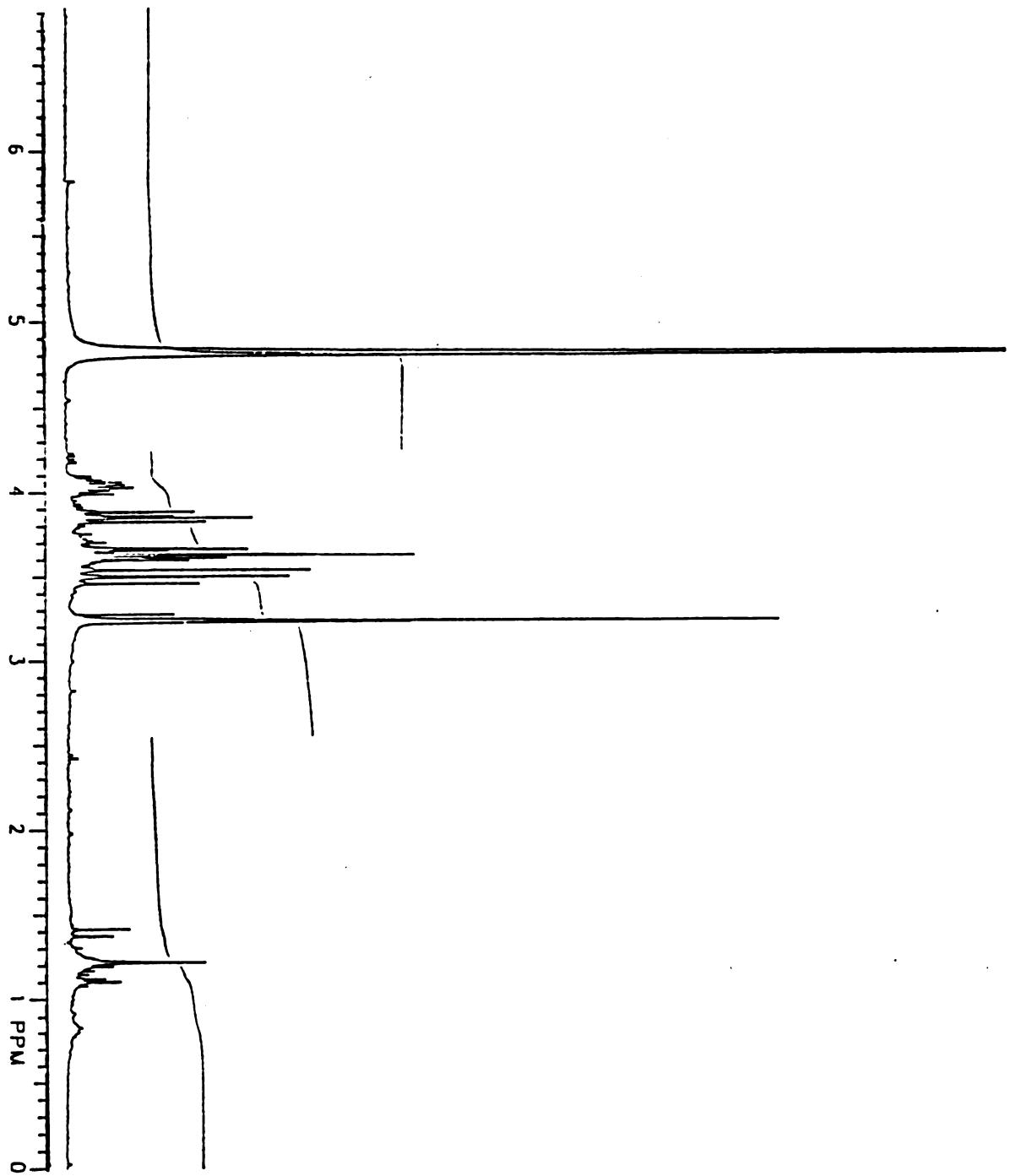
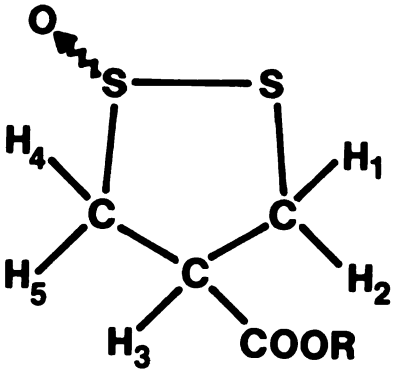

6
5
4
3
2
1 PPM
0

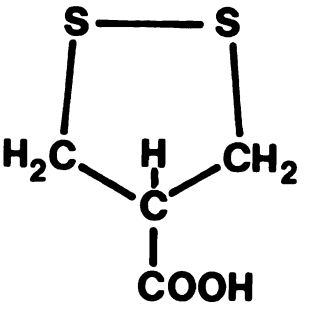
Figure 1: Chemical structures of plant growth regulators and nematicides isolated from etiolated asparagus shoot and root tissue. A = asparagusic acid-1-oxide; B = asparagusic acid; C = S-acetyldihydro asparagusic acid; and D = dihydro asparagusic acid.

A

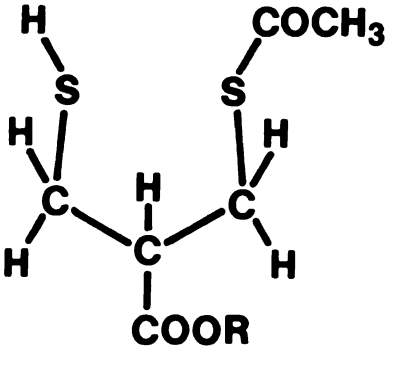



R = H
R = $CH_3$

B

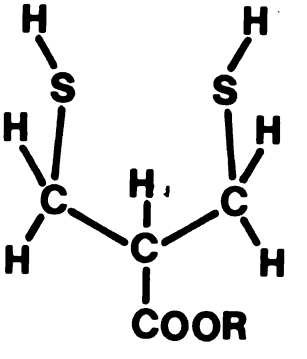



C




R = H
R = $CH_3$

D




R = H
R = $CH_3$

showed the silylated methylated molecular ion at m/z=222, and a fragmentation pattern with a peak at m/z=189 indicating a loss of an SH group, and m/z=189, a loss of the silylating group and 1 hydrogen, m/z=129, a rearrangement, and the base peak at m/z=73, loss of the silylating group (Figure 7). This fragmentation pattern is suggestive of asparagusic acid, 1,2-dithiolane-4-carboxylic acid or 1 of its other dirivatives. From data available, it was not possible to distinguish completely which dirivatives are actually present.

**Purification of inhibitory components from dried asparagus root tissue: Scheme #3.** When a crystallized component from the last ether fraction was subjected to $^{1}H$ NMR in deuterated methanol, the spectrum revealed a singlet at 1.30, 3.30 and 4.92 due to solvent impurities, undeuterated methanol and partially deuterated water, respectively. The other protons of this compound appeared at 7.53 (1 H, d, J=15 Hz), 7.01 (1 H, d, J=2 Hz), 6.92 (1 H, dd, J=8 Hz, J=2 Hz), 6.78 (1 H, d, J=8 Hz), and 6,21, (1 H, d, J=15 Hz) (Figure 8). These data suggest the structure was that of caffeic acid, 2,4-dihydroxycinnamic acid. When a standard of caffeic acid was run under the same conditions in the $^{1}H$ NMR, the spectra of the known standard and the purified unknown from the ether fraction matched exactly (Figure 9).

When the caffeic acid standard was compared to the last ether extract and the C18 crude active fraction, $I_{50}$ levels were 1.98 mg, 0.25 mg, and 0.03 mg respectively. Bioassays of all fractions from this isolation procedure show inhibitory activity only in the last ether fraction.

Figure 7. GC-Mass spectrum of a compound isolated from asparagus root tissue using preparative TLC, mobile phase, chloroform:methanol 8.5:1.5. Compound at Rf=0.07 was scraped from the plate and eluted from the silica gel with chloroform:methanol 5:1.

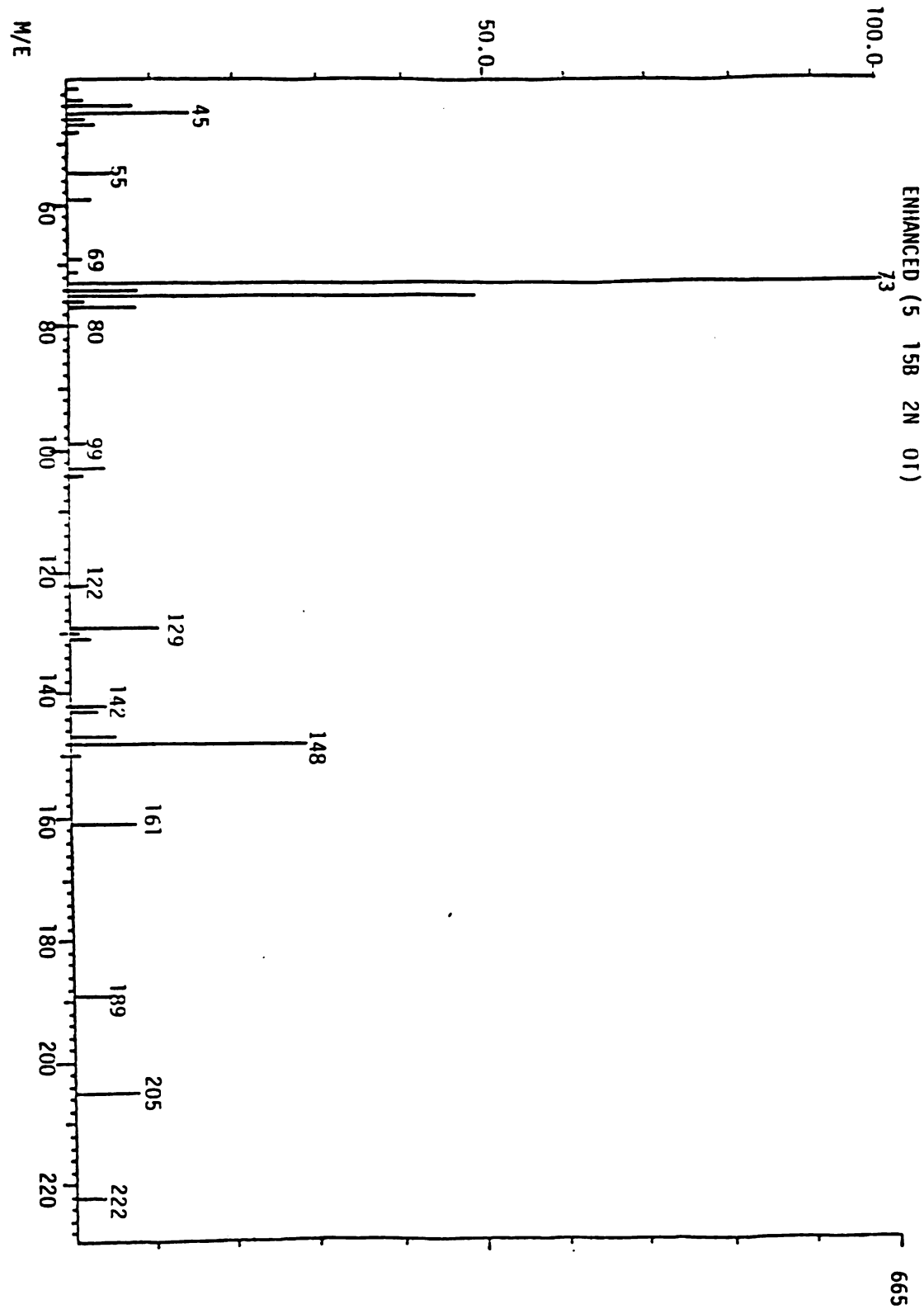

MASS SPECTRUM
10/22/86 13:28:00 + 6:54
SAMPLE: BAB/P569
CONDS.: DB5 100-250 15DEG/MIN
ENHANCED (5 15B 2N 0T)
DATA: BB26 #207
CALI: CA1022 #4
BASE M/E: 73
RIC: 2008.
100.0
50.0
M/E
45
55
60
69
73
80
80
99
100
120
122
129
140
142
148
160
161
180
189
200
205
220
222
665.

Figure 8. $^{1}H$ NMR spectrum of compound x isolated from asparagus root tissue using soxhlet extraction. The crystalline compound was dissolved in deuterated methanol.

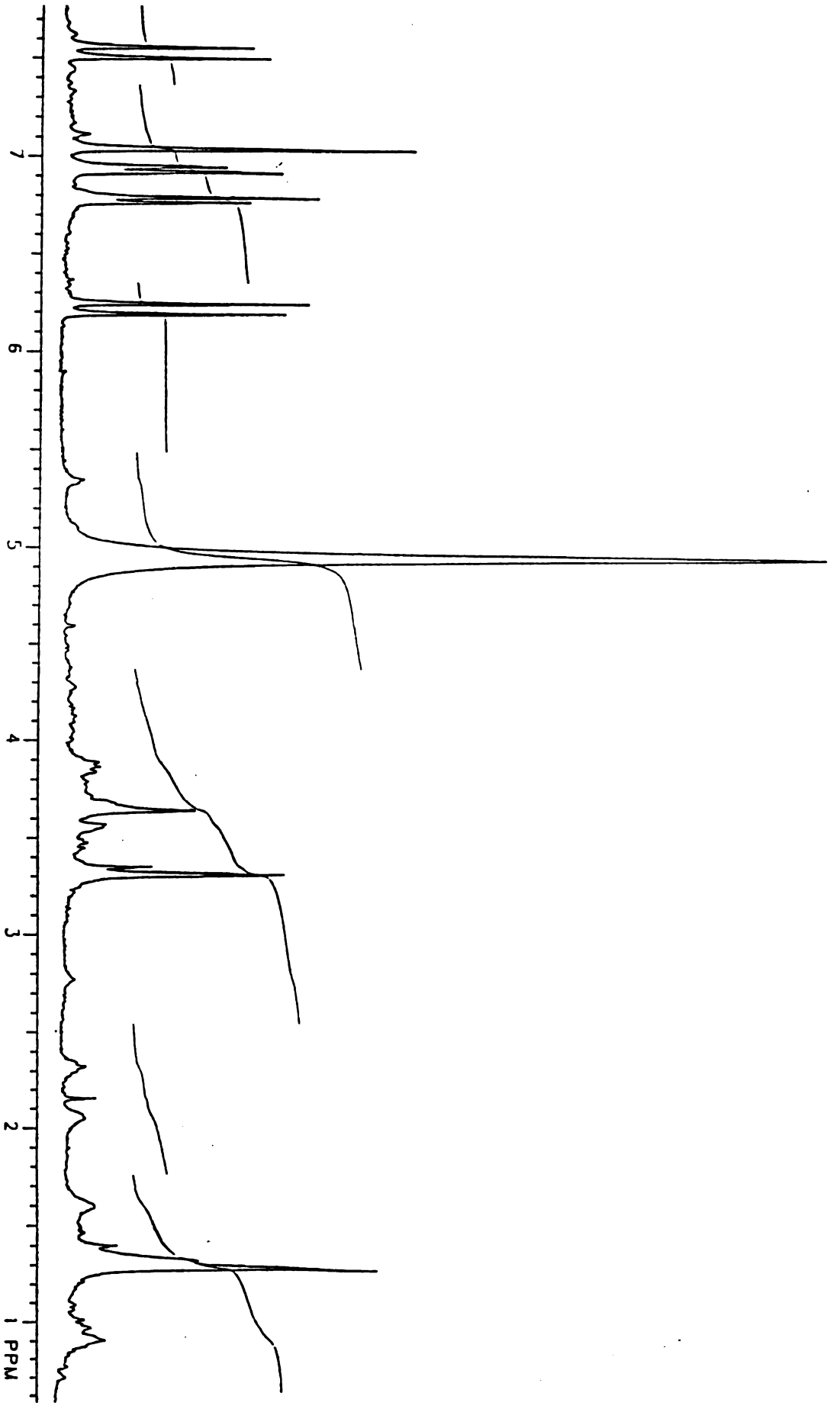
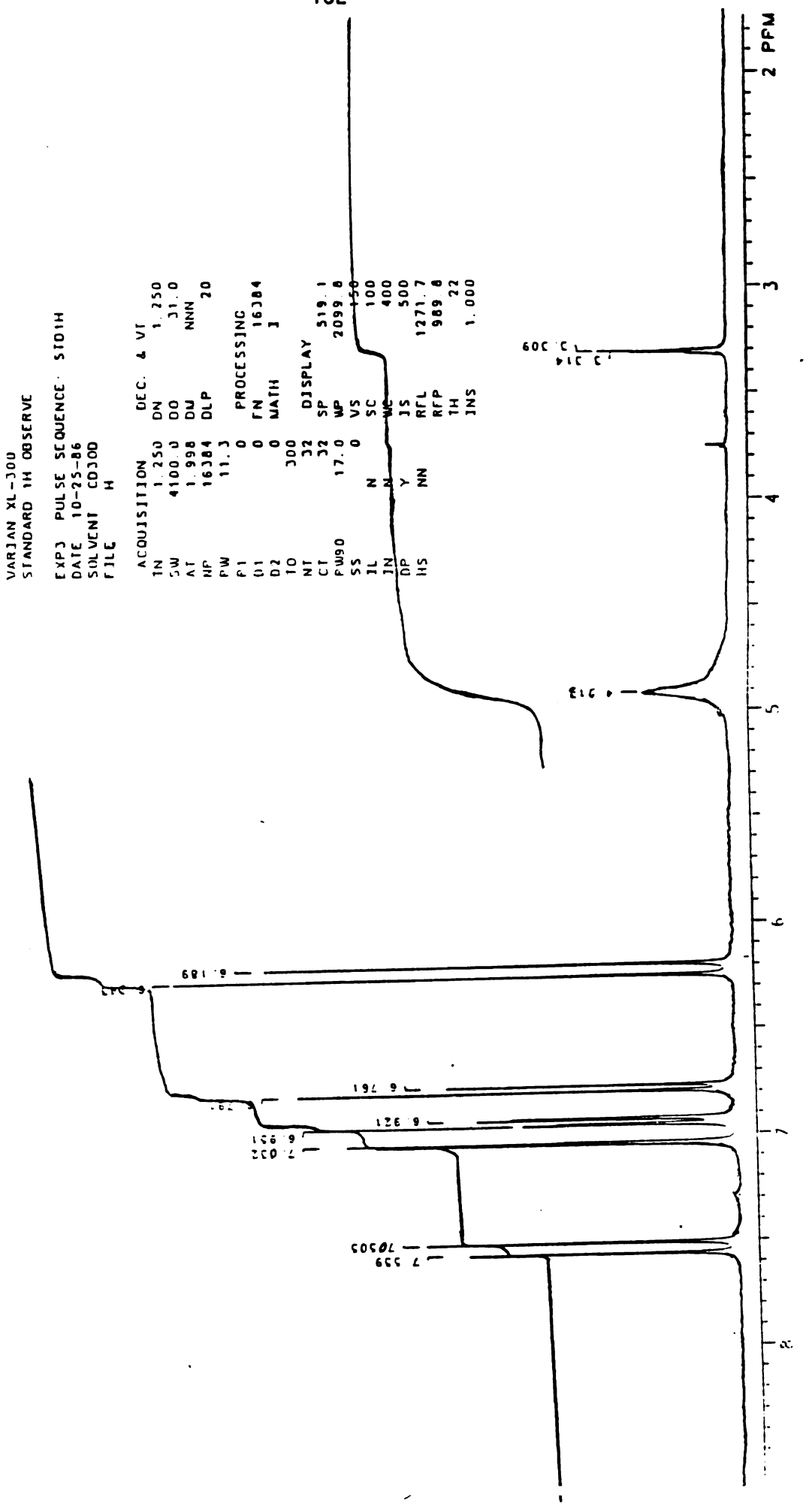

7
6
5
4
3
2
1 PPM

Figure 9. $^{1}H$ NMR of a known standard of caffeic acid dissolved in deuterated methanol.


VARIAN XL-300
STANDARD 1H OBSERVE
EXP3 PULSE SEQUENCE: SIDIH
DATE 10-25-86
SOLVENT CD3OD
FILE H
ACQUISITION
SW 4100.0
AT 1.998
NP 16384
PW 11.3
D1 0
D2 0
TO 300
NT 32
CT 32
PW90 17.0
SS 0
IL N
IN N
DP Y
HS NN
DEC. 4 VT
DN 1.250
OO 31.0
DW NNN
DLP 20
PROCESSING
FN 16384
MATH 1
DISPLAY
SP 519.1
WP 2099.8
VS 150
SC 100
WC 400
JS 500
RFL 1271.7
RFP 989.8
TH 22
INS 1.000
2 PPM
3
4
5
6
7
8

**Isolation for inhibitory components from soil.** In all bioassays on curly cress with chloroform extracts from both asparagus fields and the control soil, no inhibitory activity was noted except at concentrations of 32 mg/ml. Therefore, there were no differences in inhibitory activity from the three soils.

## DISCUSSION SECTION

Isolation of compounds from asparagus root extracts revealed the presence of several known acidic compounds that have been previously reported to be important in allelopathic interactions. Ferulic acid has been reported as a germination inhibitor produced by Camelina alyssum, and present in residues of corn, wheat, sorghum and oats. This compound has also been isolated from soils under allelopathic plants (15). Fumaric acid is a well known microbial toxin produced by Rhizopus spp. in hull rot disease on almond (13). Also, isolations suggested that asparagusic acid or one of its derivatives were present in the inhibitory fractions. These are extremely active growth inhibitors (19, 21). Caffeic acid is reported to be fungistatic against Helminthosporium carbonum in potatoes, and phytotoxic against many plant species and families (15). These compounds and the inhibitory fractions were shown to be active at varying pH levels. The fact that all inhibitory activity cannot be accounted for by pH has also been documented in the literature specifically for malic and citric acid (3). The known standards of several of the acidic compounds were shown to be inhibitory in our bioassay system but never to the extent to explain all of the activity of the crude extracts of asparagus root tissue. Also, inhibitory activity was present in other

solvent extracts other than the chloroform extract used for isolation procedures. These may have interacted in an additive or synergistic manner with the isolated compounds. Also, the cress seed bioassay may not reflect all the biological activity of the active components. Young (23) reported isolating several compounds from asparagus root exudation. These compounds were not present in any fractions from our isolation procedures. He did not test for activity of these compounds on asparagus or any other plant species. Also, since his extracts were from nonsterile sand, it is difficult to determine if these compounds were released from the asparagus root system or are products from microbial species or microbial breakdown of root exudates.

In soil extraction experiments, the methanol:water extraction procedure may have failed to remove allelochemicals bound tightly to soil particles. Also, since allelochemicals from asparagus will be active in the very narrow region of the asparagus root rhizosphere, isolation attempts from bulk soil may not be representative of chemical concentrations in that region. Allelochemicals will be active on the surface of the root, through uptake by the plant and subsequent effects on the biochemical processes of the plant, or through inhibition of beneficial rhizosphere microflora. Therefore, concentrations of allelochemicals in bulk soil may appear quite dilute.

Our experiments show that asparagus root tissue contains a number of inhibitory components important in the inhibition of seed germination and radicle elongation. These toxic components may be released from asparagus root tissue through exudation or degradation of the root tissue and may play a role in the alleged allelopathic activity of the asparagus plant. These data, as well as data presented

elsewhere (4, 5, 6) suggest that the allelopathic properties of asparagus are not due to the presence of any one chemical compound acting in isolation from other biotic factors associated with the asparagus agroecosystem, but instead is due to the presence of many chemical components that may act differently depending on soils, environment conditions and microbial populations. Further clarification of the chemicals released by asparagus root tissue, and their biological activity on asparagus plants is needed to substantiate their role in asparagus decline.

## LITERATURE CITED


1. Cohen, S. I., and F. D. Heald. 1941. A wilt and root rot of asparagus caused by Fusarium oxysporum Schlecht. Plant Disease Reporter. 25:503-609.
2. Endo, R. M., and E. C. Burkholder. 1971. The association of Fusarium moniliforme with the crown rot complex of asparagus. Phytopathol. 61:891 (Abstr.)
3. Evanari, M. 1949. Germination inhibitors. Bot. Rev. 15(3):153-194.
4. Grogan, R. G. and K. A. Kimble. 1959. The association of Fusarium wilt with asparagus decline and replant problem in California. Phytopathol. 99:122-125.
5. Hartung, A. C., and C. T. Stephens. 1984. Allelopathic properties of asparagus: Interaction with Fusarium spp. and bioassay techniques. Phytopathol. 74(7):800 (Abstr.)
6. Hartung, A. C., and C. T. Stephens. 1983. Effects of allelopathic substances produced by asparagus on the incidence and severity of Fusarium crown rot. J. Chem. Ecol. 9:1163-1174.
7. Hartung, A. C., and A. R. Putnam. 1985. Extracts of asparagus root tissues are phytotoxic. Proc. of the Sixth International Asparagus Symposium. E. C. Lougheed and H. Tiessen, eds. Univ. Guelph. Pp. 258-266.
8. Herner, R. C., and G. Vest. 1974. Asparagus workshop proceedings. Department of Horticulture. Michigan State University. Pp. 79.

9. Johnston, S. A., J. K. Springer, and G. D. Lewis. 1979. Fusarium moniliforme as a cause of stem and crown rot of asparagus and its association with asparagus decline. Phytopathol. 69:778-780.

10. Kitihara, Y., H. Yanagawa, T. Kato, and N. Takahashi. 1972. Asparagusic acid, a new plant growth inhibitor in etiolated young asparagus shoots. Plant and Cell Physiol. 13:923-925.

11. Laufer, G. A., and S. A. Garrison. 1977. The effect of asparagus tissue on seed germination and asparagus seedling growth. Possible allelopathic interaction. HortSci. 12:385. (Abstr.)

12. Lehle, F. R., and A. R. Putnam. 1982. Quantification of allelopathic potential of sorghum residues by novel indexing of Richards' Function fitted to cumulative cress seed germination curves. Plant Physiol. 69:1212-1216.

13. Mirocha, C. J., J. E. DeVay, and E. E. Wilson. 1966. Role of fumaric acid in the hull rot disease of almond. Phytopath. 51:851-860.

14. Molisch, H. 1937. Der Einfluss einer pflanze auf die audere allelopathie. Jena:Fisher.

15. Rice, E. L. 1984. Allelopathy. Academic Press, Inc. Pp. 422.

16. Shafer, W. E., and S. A. Garrison. 1980a. Effects of decomposing asparagus root tissues on lettuce, tomato, and asparagus seed emergence. HortSci. 15:406 (Abstr.)

17. Shafer, W. E., and S. A. Garrison. 1980b. Effects of asparagus root extracts on lettuce and asparagus seed germination and growth. HortSci. 15:406-407 (Abstr.)

18. Shafer, W. E., and S. A. Garrison. 1986. Allelopathic effects of soil incorporated asparagus roots on lettuce, tomato, and asparagus seedling emergence. HortSci. 21(1):82-84.

19. Takasugi, M., Y. Yachida, M. Anetai, T. Masamune, and K. Kegasawa. 1975. Identification of asparagusic acid as a nematicide occurring naturally in the roots of asparagus. Chem. Lett. Pp. 43-44.

20. Yang, H. 1982. Autotoxicity of Asparagus officinalis L. J. Am. Soc. Hort. Sci. 107:860-862.

21. Yanagawa, H., T. Kato, and Y. Kitahara. 1972. Asparagusic acid, dihydroasparagusic acid and S-acetyldihydroasparagusic acid, a new plant growth inhibitors in etiolated young Asparagus officinalis. Tetrahedrom Letters. 25:2549-2552.

20. Young, C. C. 1984. Autotoxication in root exudates of Asparagus officinalis L. Plant and Soil 82:247-253.

21. Young, C. C. 1986. Autotoxication of Asparagus officinalis. in: The Science of Allelopathy. A. R. Putnam and C-S Tang, eds., John Wiley and Sons, Inc. Pp 317.

# CHAPTER VI

## SUMMARY AND CONCLUSIONS

Asparagus, like many other perennial crops is afflicted with autotoxicity and replant problems. Classical studies that have addressed similar problems reveal that they are complex and may involve an array of pathogens, pests, nutritional factors and allelochemicals. The asparagus decline problem also appears to be complex and involves the crown and root rotting organisms, Fusarium oxysporum f.sp. asparagi and F. moniliforme. These diseases are believed to ultimately cause plant death.

This dissertation indicates that a series of other factors may interact with the asparagus plant or the pathogens to make the plant more susceptible to disease. For example, when asparagus root tissues or extracts are present, young asparagus plants become more susceptible to infection by F. oxysporum f.sp. asparagi and F. moniliforme. Since asparagus root extracts induce electrolyte leakage, decrease peroxidase activity, and decrease respiration in young asparagus roots, it may be hypothesized that compounds present in these extracts adversely affected the cell membranes making them more susceptible to invasion by the Fusarium organisms. Decreased peroxidase activity in particular, has been related to increased disease incidence in other species.

The addition of asparagus root and fern tissues to soil produces toxicity not only on asparagus, but on a variety of dicotyledon and monocotyleton indicators. Since asparagus seedlings germinate and grow so slowly, another bioassay species was needed to quickly assess the toxicity of asparagus allelochemicals. Curly cress (Lepidium sativum) proved to be a reliable species for this purpose. When asparagus tissues or extracts were incubated under aerobic or anaerobic conditions, their toxicity was generally decreased as compared to the toxicity of freshly prepared tissues or extracts. This indicates that over the short term, soil microbes tend to be detoxifiers other than producers of toxins. This is in contrast to other cases of allelopathy, particularly those involving cyanohydrin compounds, where microbes are necessary to release the toxins.

Asparagus tissues in the soil had pronounced influences on the soil microbial dynamics. Most importantly, the Fusarium species rapidly increased whenever root tissues were present in the soil. In contrast, Pythium and Bacillus species were strongly inhibited by isolated fractions from asparagus root extracts. The compounds found to inhibit the microbial species may be the same or different from those important sseedling inhibition. The data suggest that several compounds are important in the inhibitor interactions. In general, the bacterial and fungal populations were considerably lower in soils where asparagus grew in contrast to soils containing snapbeans and corn. It is hypothesized that the compounds released by asparagus shift the microbial balance in the rhizosphere in favor of the Fusarium organisms. This may occur through a reduction of their competitors or because they are better able to utilize the substrates in asparagus

tissue. In any event, the asparagus rhizosphere creates an environment especially suited for growth of Fusarium species.

Several biologically active compounds were isolated from asparagus root tissues. In any isolation procedure, the investigator must make arbitrary decisions about which fractions to follow. Some fractions with lower specific activity may receive lower priority, but should not be totally ignored. Since the chloroform fraction displayed the highest specific activity in our fractionations, these compounds were isolated first. A variety of organic acids were isolated in this fraction. The TCA cycle acids, citric, malic, and fumaric are commonly implicated in allelopathy. They are common seed germination and seedling growth inhibitors in fruits and herbage of many crops. Release of high quantities of these materials around asparagus roots could produce a high acidity condition in the rhisozphere. The ferulic and isoferulic acids found in root tissues have often been implicated in allelopathy. The former compound is persistent enough in soil to be implicated as a toxicant from several crop residues. Ferulic acid is one of the most frequently mentioned allelochemicals that is isolated from soil. Soxhlet extraction of asparagus tissue also removed caffeic acid, which is a known inhibitor of higher plant and fungal growth. Caffeic acid is particularly toxic to the root growth of germinating seedlings and might influence the susceptibility of young asparagus roots to disease invasion.

The NMR spectra suggested that a derivative of asparagusic acid or asparagusic acid-i-oxide was present in the root tissue. These compounds were previously reported to be highly phytotoxic and nematocidal. Since none of the other isolated chemicals showed activity high enough to account for the high activity of the semi-

purified extracts, these compounds or their analogs may contribute considerable activity. Further chemistry work should be focussed on these compounds and how they might interact with the other allelochemicals from asparagus roots. Once these compounds and their activities are well characterized serious attempts through trapping or extraction must be directed toward their isolation from the asparagus rhizosphere. This will provide more convincing proof of their role in allelopathy in the asparagus decline syndrome that occurs in the field.